ABSTRACT

Title of Dissertation: FENTON & FENTON-LIKE REACTIONS: THE NATURE OF OXIDIZING INTERMEDIATES INVOLVED

Nixon Ogendi Mwebi, Doctor of Philosophy, 2005

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Accumulating evidence indicates that reactive oxygen species (ROS) such as hydroxyl radical and peroxy radicals are involved in the pathophysiology of aging and a multitude of diseases such as cancer, as well as neurodegenerative disorders. These ROS are believed to result in part from Fenton and Fenton-like reactions mainly involving reactions between metal ions or metal complexes and hydrogen peroxide. The exact nature of oxidizing intermediates involved in these Fenton reactions remains uncertain and is still a matter of considerable interest. Although many studies have been conducted examining the nature of these intermediates, a

number of these have been inconclusive due to experimental artifacts in the analytical techniques involved.

A highly sensitive and selective technique is hereby employed to quantify the magnitude of species involved in these reactions. The intermediate species involved in the Fenton and Fenton-like reactions have been shown to react with both dimethylsulfoxide (DMSO) and (for the first time) methane to yield methyl radicals. A comparison of the product yields in the DMSO and methane experiments performed under identical conditions yield product ratios close to those expected for free hydroxyl radical (OH). Competition studies between the primary OH scavengers, DMSO or methane with nitroxide and benzoic acid yield product distributions typical of those expected for free OH, clearly indicating free OH involvement.

Free OH involvement is manifested both at acidic pH (4.2) and neutral pH (7.4) in Fenton reactions with both organic and inorganic metal complexes of iron. The yield of product II associated with free OH is found to decrease with increasing pH possibly due to competing side reactions. Fenton reactions in the presence of ferric ions indicate that the species formed in the reaction of OH with Fe (III), (possibly ferryl species, Fe^{IV}) oxidize DMSO to yield methyl radicals, but fail to oxidize methane, thereby providing a simple method to discriminate between free OH and the high valent metal species.

FENTON & FENTON-LIKE REACTIONS: NATURE OF OXIDIZING INTERMEDIATES

By

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2005

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DEDICATION

To my late father

Pr. Simion Mwebi Makori

ACKNOWLEDGEMENTS

I sincerely thank my advisor Dr. Neil V. Blough for his expert guidance, intellect and support throughout this period of study. Dr. Blough, your patience is exceptional and from you I have learned not only knowledge but discipline, kindness, and professionalism. These values I highly appreciate and treasure and believe will aid me in my future endeavors.

I also want to thank Dr. George R. Helz for his continual advice, valuable inputs, support and most of all encouragement. Dr. Helz, your time, continual care and understanding made it all bearable. I sincerely thank Dr. Fenselau for her continual encouragement and believing in me from the very first time I joined the program. Thank you for your advice and guidance all along. I also want to thank Dr. Sang Bok Lee and Dr. Allen P. Davis for their guidance, and ready assistance. My sincere thanks and gratitude also goes to Dr. Steven Rokita for his advice and very valuable inputs in this work.

I want to thank the Blough group members; Trudy, Rossana, Matt, Gan, Pramila, Marjan, Qing, Natasha, Min, Sarah and Mati for their encouragement and friendship over the years.

I sincerely thank my family: my beloved mother Mogute and my brothers and sisters, for their support. Your love and prayers kept me going. Mum, thank you for believing, waiting and encouraging me to finish further studies that you least understood.

Further thanks go to my dear friends and mentors; Dr. Urasa, Dr. Chacha and Mr. Obiri, you all helped lay the foundation for this venture. Special thanks to my dear

brother-in-law Dr. Obare for his invaluable encouragement and input over the years, you really made me realize I can do it. I also thank Wamuyu and Mogoi for their invaluable help in editing this manuscript.

Special thanks go to my “church family” Capital Chinese Church for their warmth and friendship. I especially thank Pr. Jiao for spiritual guidance and sister Concepta, for being at home with our family at the right time. Most important, I want to thank my “Mom”, Mrs. Suki, and her family for their immensurable help. Your willingness to take care of issues outside the class, really gave me the motivation and time to do it all.

Finally, much thanks goes to my dear family, my darling wife Phane and children Audrey and Brian. This Ph.D. is as much mine as it is theirs. Phane, thank you for your patience, understanding, support and friendship over the years. I don’t know how I could ever have done it without you. Brian and Audrey, I most heartedly thank you for your love and understanding, during all those missed play hours at the park and at home without me. You guys endured it all without complains but with great understanding and lots of prayers, your attitude was my motivation and you are the best I can ever ask for.

Lastly, I join Albert Einstein in thanking a God with whom there are no accidents, for guiding me through this part of my life safely and bringing into my life such a wonderful persons as these. I believe that He makes all things beautiful in His time.

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LIST OF ABBREVIATIONS

BA	Benzoic acid
EDTA	Ethylenediaminetetraacetic acid
NTA	Nitrilotriacetic acid
DTPA (DETAPAC)	Diethlylenetriamine pentaacetic acid
DMSO	Dimethyl sulfoxide
DMPO	5,5, Dimethyl Pyrroline-N- oxide
EPR	Electron Paramagnetic Resonance Spectroscopy
FLRS	Fenton-Like Reactions
GC-MS	Gas Chromatography- Mass Spectrometry
3AP	3-Amino-2, 2, 5, 5-tetramethyl-1-pyrrolidinyloxy
3CP	3- Carbamyl pyrrolidinyloxy
2-OHBA	2- hydroxylated benzoic acid
HO	hydroxyl radical
$R^{\cdot}$	Alkyl radical species
$HO\text{-}Ar^{\cdot}$	Aryl radical species
$e^{-}_{(aq)}$	Hydrated electron
k	Rate constant
UV-VIS	Ultraviolet visible spectroscopy
HPLC	High performance liquid chromatography

CHAPTER I

Fenton & Fenton-Like Reactions: Nature of Oxidizing Intermediates

1.1 Introduction

The Fenton reaction defines the reaction of ferrous iron with hydrogen peroxide (Reaction 1.1). This reaction was named after H. H. Fenton (1894) who first observed the oxidation of tartaric acid by a mixture of iron sulfate and hydrogen peroxide acid. The oxidant involved was originally interpreted by Barb (1951) as HO (Reaction 1.1). Fe (II) (with trace amounts of Fe (III)), is known to decompose H_2O_2 to oxygen and water. This may be understood by the following sets of reactions (1.1-1.5).

$$Fe\ (II) + H_2O_2 \longrightarrow Fe(III) + HO^- + HO^{\bullet} \qquad 1.1$$

In the absence of organics and in the presence of excess H_2O_2, the HO will react with H_2O_2 to form superoxide.

$$HO^{\bullet} + H_2O_2 \longrightarrow O_2^{\cdot -} + H^+ + H_2O \qquad 1.2$$

Originally, the superoxide was thought to react with hydrogen peroxide, in

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + HO^- + HO^{\bullet} \qquad 1.3$$

what is commonly known as the Haber Weiss reaction (Reaction 1.3). Later studies by Barb et al 1951 and Rush and Bielski 1985 have shown unequivocally that Reaction 1.3 does not take place as written, (because of the very low rate constant of the reaction). Instead, superoxide reduces Fe (III) (Reaction 1.4) rather than H_2O_2, and

$$O_2^{\cdot -} + Fe(III) \longrightarrow O_2 + Fe(II) \qquad 1.4$$

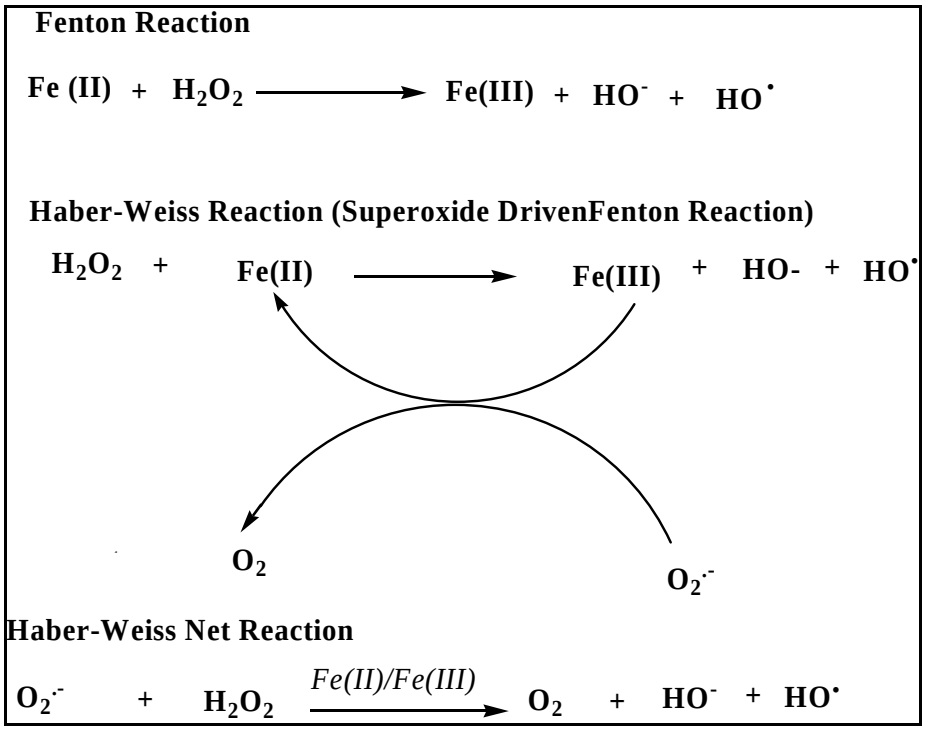
$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + HO^{\bullet} + HO^- \qquad 1.1$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + HO^{\bullet} + HO^- \qquad 1.3$$

the overall reaction is therefore seen as a decomposition of H_2O_2, (Reaction 1.5), i.e. reaction 1.3 is the sum of reactions 1.1 and 1.4.

$$2H_2O_2 \longrightarrow O_2 + 2H_2O \qquad 1.5$$

These reactions are summarized in the following Scheme1.1;



Scheme 1.1 Basic free radical mechanisms for the Fenton and Haber-Weiss Reaction, (Branchaud, B. P., 1999)

Fe (III) is also known to catalytically decompose H_2O_2 to oxygen and water in a series of reactions where the Fe (III) slowly reduces the H_2O_2 to O_2^- (Reaction 1.6) which is decomposed further to dioxygen. The Fe (II) thus formed may then react with H_2O_2 to yield HO

$$Fe(III) + H_2O_2 \rightleftharpoons Fe(II) + O_2^{\bullet-} + 2H^+$$

$$Fe(II) + O_2^{\bullet-} \xrightarrow{2H^+} Fe(III) + H_2O_2$$

$$Fe(III) \quad O_2^{\bullet-} \longrightarrow Fe(II) + O_2$$

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

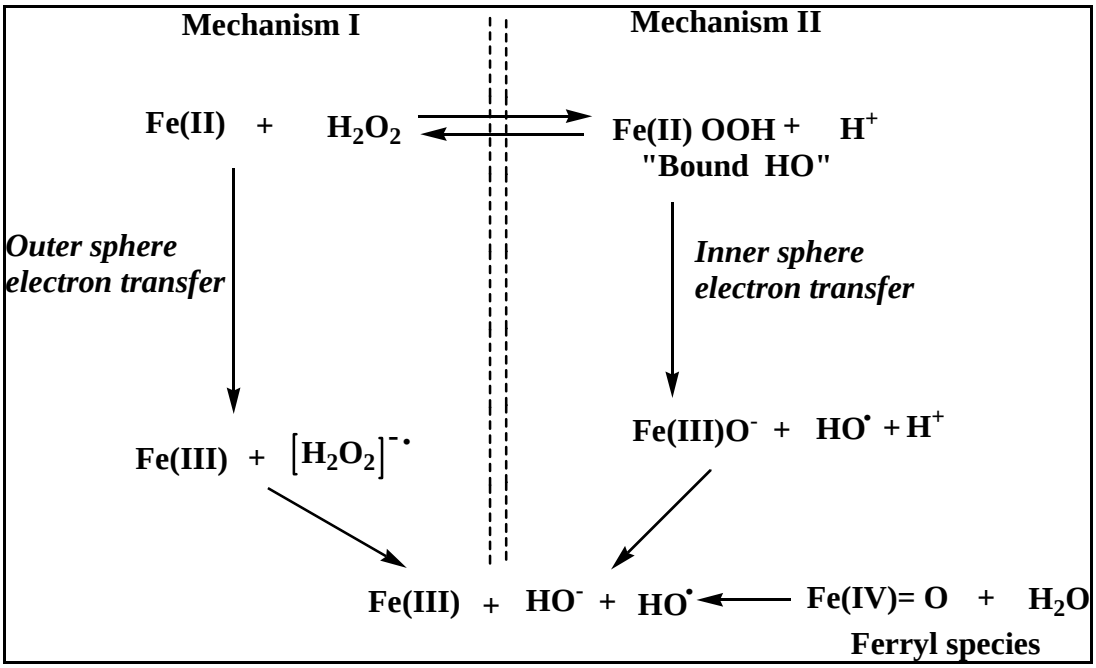
$$Fe(III) + H_2O_2 \longrightarrow Fe(II) + HO^{\bullet} + HO^{-}$$

The mechanism of the Fenton reaction has been suggested to be more complicated than the simple reaction scheme presented in scheme 1.1. Since iron has a variable valency, its oxidation by H_2O_2 may occur via a one or two electron transfer, (Reactions 1.1 and 1.9 respectively).

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + HO^{-} + HO^{\bullet} \qquad 1.1$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(IV) + 2OH^{-} \qquad 1.9$$

Some studies therefore suggest that the classical Fenton reaction occurs using only Fe (II) as an electron donor to H_2O_2. Such would be an outer sphere electron transfer reaction with no direct bonding interactions between the electron donor and the acceptor, (Mechanism I, Scheme 1.2), (Cannon R. D., 1980). On the other hand, recent studies have shown and favored the inner sphere electron transfer mechanisms, which involve direct bonding between the iron and H_2O_2. This interaction could produce a metal-peroxo complex, Fe (II) HOO which may react further to generate either HO radicals (one-electron oxidant) or Fe (IV)=O (two electron oxidant), (Masarwa et al 1988), (Mechanism II, scheme 1.2). The key question therefore is which of these species is the major oxidant in these reactions.



Scheme 1.2 Basic reactions and intermediates involved in the classic Fenton and the metal centered Fenton reactions (Branchaud et al 1988)

Even in autoxidation reactions where the ferrous metal reacts with oxygen, (Reaction 1.10), the nature of the oxidizing species involved is still a matter of considerable debate. Although HO has long been thought to be the species involved in the ferrous autoxidation reactions (Reactions 1.10 - 1.18, below), a number of other studies suggest involvement of ferryl (Fe^{2+}-O) or perferryl (Fe^{2+}- O_2 or Fe^{3+} - O_2) species, (Reactions 1.11 – 1.14), (Qian and Buettner 1999, Urbanski and Beresewicz 2000).

$$Fe^{2+} + O_2 \longrightarrow Fe(III) + O_2^{\cdot -} \qquad 1.10$$

$$2O_2^{\cdot -} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad 1.8$$

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + H\dot{O} + HO^- \quad 1.1$$

$$Fe^{2+} + O_2 \longleftrightarrow [Fe^{2+}\text{-}O_2 \longleftrightarrow Fe^{3+}\text{-}O_2] \longleftrightarrow Fe^{3+} + O_2^- \quad 1.11$$

$$Fe(III) + O_2^{\cdot -} \longrightarrow O_2 + Fe(II) \quad 1.4$$

$$Fe\ (II) + H_2\dot{O}_2 \longrightarrow Fe^{2+}O + H_2O$$

1.12

The ferryl species may be further generated by the reaction of the perferryl ion with another ferrous ion, (Reactions 1.13 and 1.14).

$$Fe^{2+} + Fe^{2+}\text{-}O_2 \longrightarrow Fe^{2+}\text{-}O_2\text{-}Fe^{2+}$$

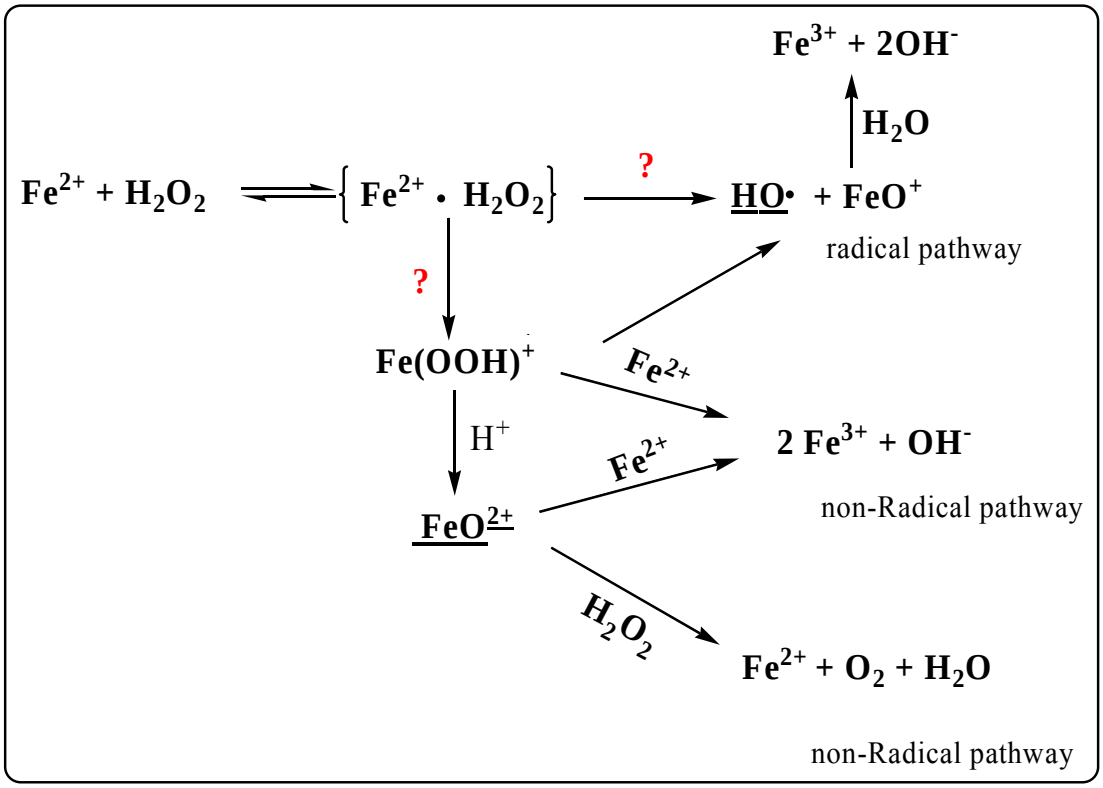
1.13

$$Fe^{2+}\text{-}O_2\text{-}Fe^{2+} \longrightarrow 2(Fe^{2+} - O) \quad 1.14$$

The perferryl species which are thought to be important in biological systems have high electron affinities and are therefore assumed to be able to exhibit reactivities approaching that of HO (Qian and Buettner 1999).

The mechanism of the Fenton reaction may therefore be even more complicated than represented by the basic forms in Scheme 1.1 or 1.2. Various pathways have been proposed (Meyerstein et al 1999, Walling 1998, Kremer 1999, Sawyer et al 1996)

including: non-radical mechanisms, radical mechanisms involving oxygen centered radicals and reactions of high-valent metal species (Scheme 1.3). Due to the importance of Fenton reactions in biological and environmental systems elucidation of the nature of species involved in these reactions has been the subject of many studies. These studies, as discussed herein, have been carried out at varying pH using both organic and inorganic metal complexes and employing a variety of free radical analysis techniques which are usually indirect.



Scheme 1.3 Possible reaction pathways for the Fenton Reaction in absence of organic substrates

Analogous systems, employing , several metals (such as Cu, and Cr or Fe (III), have replaced the ferrous metal and other peroxides have replaced hydrogen peroxide. In

such instances, the reactions are known as Fenton-like reactions. Fenton-like reactions involving metals in high oxidation states e.g. Fe (III) are also known as superoxide driven Fenton reactions and are typical of the metal dependent Haber-Weiss reaction (Scheme 1.1).

1.2 Studies with Inorganic iron complexes

A number of studies have supported the involvement of HO in Fenton reactions involving inorganic metal complexes. For example, a study of the Fenton reaction performed in presence of 2-propanol, indicate formation of oxidizing intermediates that oxidize the 2-propanol (possibly HO), even in excess of peroxide. Similar studies with alcohol have found that the total concentration of organic radicals is unchanged when excess H_2O_2 is used (Czapski, 1971).

Studies in acidic pH (0-2), in presence of organic substrates such as acetylene, carbonyl compounds, and unsaturated fatty acids have found that under conditions of excess metal ion, the Fenton reaction generates HO which attacks the organic substrates and initiates a chemical chain reaction (Walling et al, 1973, 1974 and 1975). The inability of the species involved to display a dependence on ionic strength, further indicate that the species involved are uncharged (Waling et al, 1974), confirming them to be HO.

The Fenton reaction has been found to be the only source of free radicals in an aqueous reaction containing Fe (II)/Fe (III) and H_2O_2, and in cases where Fe (III) are involved, the $HO_2^-/O_2^{\cdot-}$ couple reduces the ferric metal complex to the ferrous state where it can react with H_2O_2 in a Fenton reaction, typical of the metal-driven Haber Weiss reaction (Scheme 1.1), (Rush and Bielski 1985). A re-examination of this study (Rush

and Bielski 1985) by Kremer (2000), concluded that under the conditions of experimentation, free radicals play no part in the system, and that FeO^{2+} ions are the active intermediates. A comparison of the products formed from Fenton's reagent with those from radiolysis in presence of N_2O and DMSO, (Eberhardt and Colina (1988), has shown that, in both cases, HO radicals are formed as evidenced by the quantitative conversion of DMSO to methyl radicals and further to CH_4 and C_2H_6.

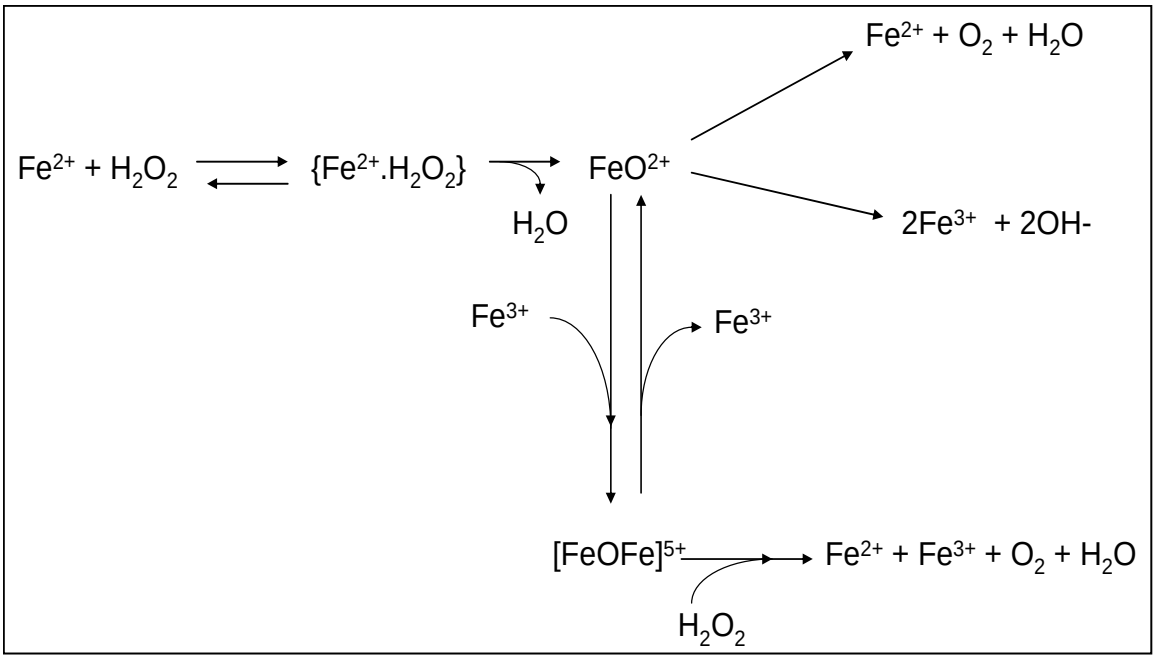
Other studies have vehemently supported the involvement of high-valent metal species in Fenton reactions with inorganic metal complexes. Such studies (Rush and Koppenol 1991) with ferrous phosphates have shown that under conditions of excess H_2O_2 the dependency of the reaction rate was inconsistent with HO. Studies at alkaline pH (8.5-12.5) have indicated that the hypervalent ion species Fe (V) and Fe (VI) can react preferentially with the protonated form of amino acids in the presence of oxygen and participate in the one-electron oxidation of organic compounds leading to radical chain reactions. This led Shepherd et al, (1991) to the conclusion that presence of chain reactions is not conclusive proof of HO involvement as previously thought.

A detailed product analysis (Sawyer et al, 1996) using GC-MS of the reaction of Fe(II)-H_2O_2 in presence of organic ligands such as picolinic acid $(PA)_2$, and pyridine have suggested that Fenton reagents do not produce HO, free carbon radicals ($R^{\cdot}$) or even free aryl adducts ($HO\text{-}Ar^{\cdot}$). Such studies have justified their assertions with further claims that no one has been able to get Fenton and Fenton-like reagents to react with methane as is expected for HO. It was further concluded (Bossman et al, 1998) that at pH 3.0 and excess peroxide, high valent metal intermediates are involved in both thermally and photochemically enhanced Fenton reactions. This observation has been supported by

studies of the photo assisted Fenton reaction ($Fe^{3+} + H_2O_2 + hv$) at pH 2.8 and excess peroxide (Pignatello et al 1999), which claim presence of a transient species (possibly ferryl) formed by decomposition of $Fe(O_2H)^{2+}$ complex, formed by the interaction of Fe^{3+} and H_2O_2. The transient species was claimed to have characteristics different from those of HO but could also be a precursor of HO.

A review by Dunfold, (2002), based on the work of Kremer (1999), (Scheme 1.4) argues that: (1) in the Fenton chemistry, the reaction of Fe(II)-H_2O_2 involves no free radicals and that the extensive radiation chemistry data on elementary reactions of oxy-radicals while undoubtedly true is irrelevant to Fenton chemistry, (2) the Haber Weiss reaction does not take place as agreed by many and, (3) whereas HO radical is expected to react with methane, no one has been able to get a Fenton-like reagent nor a heme ferryl species to react with methane. Similarly, recent static DFT calculations (Ensing et al, 2003) on the hydrated iron (III) complexes in vacuo, predicts that the ferryl ion is easily formed when H_2O_2 co-ordinates to iron in water (Reaction 1.15).

$$Fe^{2+} + H_2O_2 \quad ========\rightarrow \quad [Fe^{IV}O]^{2+} + H_2O \qquad 1.15$$



Scheme 1.4 Proposed non-radical mechanism for the Fenton reaction (Kremer 1999)

1.3 Studies with organic metal complexes

Ligands can influence rates of metal ion reactions in two ways; they may modify the reduction potential of the metal making redox cycling more, or less facile, or they may hinder access of dioxygen or reductants to inner sphere electron transfer (Miller et al, 1990, Welch et al, 2002). Those ligands that preferentially bind either the oxidized or reduced form of the metal will alter the reduction potential. Thus, a chelate like EDTA which decreases the reduction potential of iron from $E^0 = 0.11V$ to $E^o = 0.12V$ accelerates autoxidation of ferrous iron whereas, disferrioxamine which binds ferric ions stronger than the ferrous ions and decreases the reduction potential of iron further to $E^o =$

-0.45, strongly inhibits redox cycling of the iron (Bandy et al, 2001). Ligands are usually employed in metal systems because they form stable water-soluble complexes with multivalent metal ions, while preventing undesirable interactions. Generally, the larger the number of co-coordinating groups to a metal atom the more stable the resulting complex. Ligands most commonly examined for the Fenton reaction are; ethylenediaminetetraacetic acid, (EDTA), diethylenetriaminepentaacetic acid (DTPA) and nitrilotriacetic acid (NTA), (Figure 1.1).

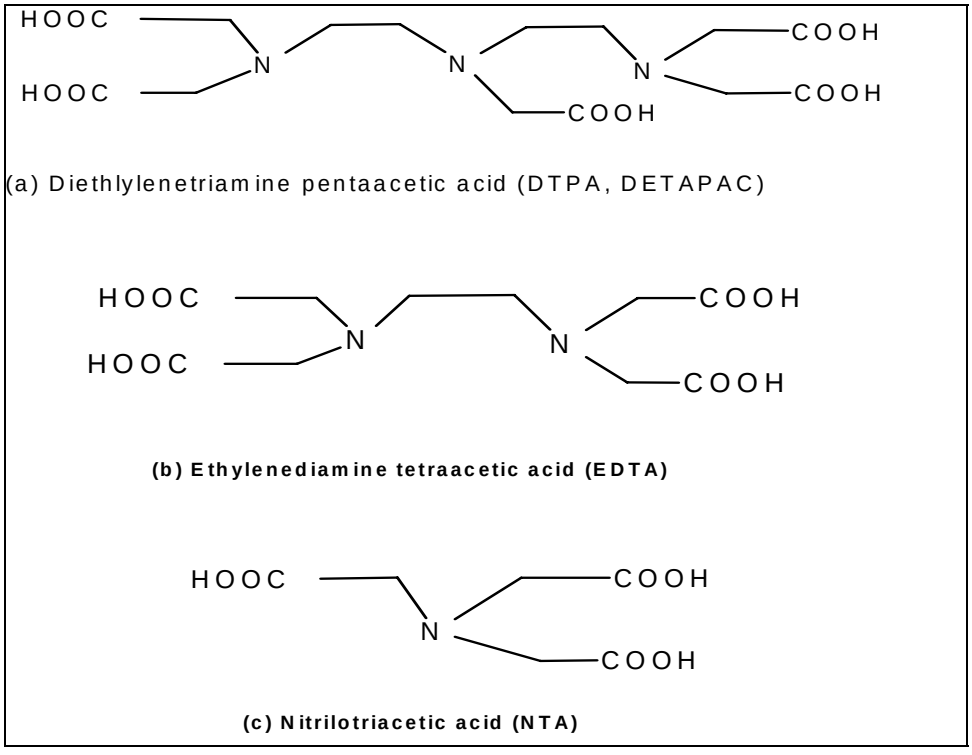


Figure 1.1 Structures of the polyaminocarboxylic acids commonly examined in the Fenton reaction

1.3.1 Ethylenediaminetetraacetic acid

As early as 1970, Cheves Walling and colleagues investigated the iron (II)-EDTA-peroxide system by spectrophotometric measurements and found that the complex formed in the reaction decomposed hydrogen peroxide and also yielded oxidation products (with organic substrates such as ethylene glycol, methylene glycol or even the EDTA ligand itself) that resembled those brought about by the hydroxyl radical. This led them to propose that the species involved was HO. Since then, several other studies have supported the involvement of HO in Fenton or Fenton-like reactions involving the iron-EDTA complex.

Studies using xanthine/xanthine oxidase as a superoxide source (Halliwell, 1978), in the presence of Fe (III)-EDTA have demonstrated the superoxide-dependent formation of hydroxyl radicals in the presence of the iron chelate. The intermediacy of free HO was detected by hydroxylation of salicylate and was further confirmed by inhibition of phenol formation by HO radical scavengers such as mannitol. These results were in agreement to the superoxide driven-Fenton reaction (Scheme 1.1) and with studies by Buettner et al, 1983 on the same reaction which indicated that introduction of HO scavengers caused significant inhibition of the HO generation indicating that the iron-chelate complex (Fe-EDTA) is capable of participating in the Fenton reactions.

Similar studies (Eaton et al, 1984) have found that for the iron-catalyzed hydroxyl radical formation to be effective, there is a stringent requirement for a free iron co-ordination site to be available. Therefore, chelates like EDTA and NTA in which a co-

ordination site on iron remains open or loosely associated with ligands such as water are efficient catalysts for HO generation.

Studies with deoxyribose (Gutteridge et al, 1990) indicate superoxide-dependent formation of the hydroxyl radical from the ferric complexes of EDTA and hydrogen peroxide, as evidenced by damage that is not inhibited by the free radical scavenger mannitol. Similarly, studies with low-valent metal complexes (Meyerstein et al, 1995) have shown that at pH < 5.5, the Fe (II)-(EDTA)-H_2O_2 intermediate decomposes to yield HO radicals, which react with organic substrates by β-hydrogen abstraction.

Other studies with Fe-EDTA have supported the involvement of high-valent metal species. For example, the intermediates involved in the Fe(III)-EDTA /H_2O_2 reaction have been found to react further with H_2O_2 to form a secondary intermediate that is found to be much less reactive towards the benzoate ion but readily oxidizes ethanol, purporting it to be non-radical (Rush et al, 1990). Similarly, recent studies of the Fenton reaction using 5, 5, dimethyl pyrroline-N-oxide (DMPO) in presence of ethanol and telephthalic acid (TA) indicate formation of an oxidizing intermediate different from HO (Welch et al 2002).

It has also been suggested that both HO and high valent metal species may be involved in Fenton reactions with the Fe-EDTA complex. Yet EPR studies of both the photolysis of H_2O_2 and the Fenton reaction (Fe (II)-EDTA/H_2O_2) in the presence of DMPO and ethanol suggest that ferryl species are involved in the Fe-EDTA reaction and that the nature of species changes from ferryl to HO with increasing concentration of the peroxide. The study further concludes that unlike the HO from photolysis which is totally

free, the HO from the Fenton reaction is only free to an extent governed by the nature of chelate used (Yamazaki et al, 1991).

1.3.2 Nitrilotriacetic acid (NTA)

In the case of Fe-NTA, Fenton reaction studies with Fe-NTA have indicated the involvement of HO, high-valent metal species or both. Indeed, studies with Fe-NTA have found it to mediate HO formation in a Fenton-like reaction driven by superoxide (Eaton et al, 1984). On the other hand, a study of the reaction of Fe-NTA and H_2O_2 (Rush and Koppenol 1988) indicate involvement of an iron species capable of oxidizing a simple carbon compound or organic ligand, in a reaction not inhibited by radical scavengers such as formate, leading to the suggestion that the species might be a hypervalent iron complex (HLFe (IV) $(HO^-)_2$ and not a hydroxyl radical. However, pulse radiolysis studies (Czapski et al, 1988) of the Fe (II)-L/H_2O_2 reaction, found it hard to distinguish between the HO radical and ferryl species using formate as the scavenger for the intermediate species.

1.3.3 Diethylenetriamine pentaacetic acid (DTPA)

In the case of DTPA, ESR studies with the spin trap DMPO have indicated that Fenton and Fenton-like reactions with the iron-DTPA complex involve HO (Egan et al, 1992). It has however also been suggested that the Fenton reaction involving Fe (II)-DTPA may not involve only HO radicals as earlier thought (Yamazaki et al, 1990), but may involve three types of species; HO, ferryl species or neutral species (Yamazaki et al,

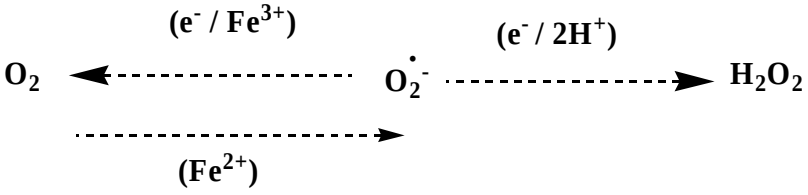
1991). This supports earlier studies by Rahhal et al, (1988) who also suggested involvement of high-valent metal species in the Fe-(III)-DTPA/H_2O_2 reaction.

1.4 Role of iron in oxidative reactions

In general, the toxicity of iron has been attributed to its ability to reduce molecular oxygen thus forming partially reduced oxygen species. For example, the ability of iron, to facilitate Fenton reactions in vivo and in vitro has been studied widely. These studies which have looked at the efficiency of these metals in mediating these reactions have varied widely in their conclusions (Sawyer et al, 1996; Kremer, 1999; Samuni et al, 1983; Yamazaki et al, 1991, and Meyerstein and Goldstein, 1998, Scheme 1.3).

Iron (II) may react with oxygen to generate superoxide (Reaction 1.16). On the other hand, iron also facilitates the conversion of O_2^- and H_2O_2 to HO or high valent metal species (Fenton and Haber–Weiss reactions). Several species may therefore arise in these oxidative reactions associated with iron and a number of these are central to the deleterious reactions in biological and environmental systems and therefore provide a means by which iron exerts its toxic effects. Of importance is the superoxide ion, the hydroxyl radical and the high valent metal species.

The superoxide though prevalent in these systems is extensively hydrated in aqueous systems where it reacts primarily through oxidation- reduction reactions (Reaction 1.13), thereby yielding hydrogen peroxide, (Fridovich, 1970).



1.16

The hydrogen peroxide thus generated serves as a precursor for the hydroxyl radical or high valent metal species in Fenton reactions. The hydroxyl radical has been found to be one of the most reactive species in its neutral form, strongly oxidizing with an HO/HO^- couple of $E^o = 1.8$ V at neutral pH. This value increases to $E^o = 2.7$ V at low pH (Buxton et al, 1999, and Bielski, 1991). The high valent metal species, mainly Fe (V) and Fe (IV) moieties are thought to constitute the catalytically active sites of iron in some biological and industrial catalysts.

Fe (V) may be obtained from Fe (VI) by reduction with a hydrated electron or by selective organic reduction (Bielski 1989, Rush & Bielski, 1987 (Reactions 1.17 & 1.18). Fe (VI) is itself obtained by synthetic oxidation of $Fe(NO)_3$ with hypochlorite in (5-10) N KOH (Bielski 1991, Thompson et al 1951) at alkaline pH.

$$Fe\ (VI) + e_{(aq)} \longrightarrow Fe(V) \qquad k = 2 \times 10^{10}\ M^{-1}s^{-1} \qquad 1.17$$

$$Fe\ (VI) + CO_2^{\cdot -} \longrightarrow Fe\ (V) + CO_2 \qquad k = 3.5 \times 10^{8}\ M^{-1}s^{-1} \qquad 1.18$$

The UV spectrum of FeO_4^{3-} at 368 nm, $\varepsilon_m = 990\ M^{-1}cm^{-1}$ has been obtained and the decay of this species primarily leads to Fe (III) and H_2O_2 (Bielski 1989).

Fe (IV) complexes with simple ligands (HO^-, $P_2O_7^{4-}$) are prepared from other Fe (III) complexes by oxidation with HO radicals (Reactions 1.19 & 1.20).

$$HO + Fe(OH)_4^- \longrightarrow FeO(OH)_n^{2-n} + H_2O/OH^- \quad k = 8.5 \times 10^7\ M^{-1}s^{-1} \quad 1.19$$

and

$$[(P_2O_7)_2Fe^{3+}OH]^{6-} + HO \longrightarrow [(P_2O_7)_2Fe^{IV}O]^{6-} + H_2O \quad k = 7.8 \times 10^7\ M^{-1}s^{-1} \quad 1.20$$

Studies of the ferryl pyrophosphate complex $[(P_2O_7)_2Fe^{IV}O]^{6-}$ have found it to be a relatively strong oxidizing agent with a reaction rate with H_2O_2 of $3.6 \times 10^5\ M^{-1}s^{-1}$ (Rush & Bielski, 1986).

It is however important to note that the high valent metal species are generally unavailable (especially at neutral or acidic pH) from an independent source. Therefore, it is difficult to demonstrate their involvement in Fenton reactions (Eberhardt, 2001). The formation or involvement of the ferryl species in Fenton reactions is therefore indirectly deduced from the presence of species having different reactivity from that of the hydroxyl radical (Yamazaki et al, 1991).

1.5 Biological and Environmental Significance

1.5.1 Biological systems

It is commonly accepted that the oxidizing intermediates involved in Fenton reactions cause damage to biomolecules and play a major role in the aging process and a variety of diseases such as cancer. The nature of the species responsible for this damage, is however still unclear, and although most studies implicate the highly reactive hydroxyl radical as responsible for the damage, other studies champion the involvement of high

valent metal species. The latter group of studies even questions the importance and occurrence of the Fenton reaction in biological systems, due to the supposedly low concentrations of H_2O_2 and "free iron" in the systems. They also claim that the high and indiscriminate reactivity of the hydroxyl radical limits its ability to diffuse and cause more extensive damage to biomolecules.

To address these issues, direct and indirect evidence of the existence of a free iron pool has been provided through several techniques (Keyer and Imlay, 1996, 1997, Cooper and Porter, 1996, Tangeras, 1985). This pool is thought to result from the malfunctioning of the complex mechanisms of uptake, storage and utilization of iron (Liochev, 1999). Studies have also shown that many reductants such as O_2^- are capable of releasing iron from ferritin (Thomas et al, 1985, Monteiro et al, 1989), and that the released iron may be capable of catalyzing free radical reactions (Carmine et al, 1995).

Similarly, high levels of hydrogen peroxide have been measured in the breath of humans (Williams 1983, Horvath et al, 1998, Nowark et al, 1996), blood plasma (Veerma & Dvanhoaran, (1991, Lacy et al, 1998, Deskur et al, 1998) and fresh urine obtained from humans (Halliwell et al, 2000). Hydrogen peroxide has also been found to liberate iron from heme proteins (Halliwell & Gutteridge, (1999), Halliwell & Gutteridge, (1990), Gutteridge, (1986), Puppo and Halliwell, (1988), making it available for oxidative reactions such as the Fenton reaction.

Many studies have been carried out in biological systems to elucidate the nature of species involved in these systems. Given the complex nature of the biological system, the involvement of radical species, for example, could be implied when presence of radical scavengers prevents damage to the biomolecules and damage is evident in their

absence (Tullis et al, 1995, Peskin et al, 1996). This has been found to be the case in studies involving organic metal complexes such as Fe-EDTA (Evans et al, 2000 and Fojta et al, 2000) and Fe-NTA (Aruoma et al, 1989, Liebold et al, 2002 and Hamazaki et al, 1989). In cases where the damage is not inhibited by radical scavengers, a site specific mechanism has been suggested, (Samuni et al, 1983, Czapski et al, 1983) or the possible involvement of "crypto HO", (bound HO), (Saran 2000) or high valent metal species.

1.5.2 Environmental systems

The Fenton reaction is often named as a possible source of HO in sunlit waters (Moffet et al, 1993, Zepp et al, 1992). Other sources include photolysis of nitrite (Zafiriou et al, 1987), nitrate (Zepp et al, 1987), metal to ligand-charge-transfer reactions (Faust, 1994), photo-Fenton reactions (Zepp et al, 1992), as well as dioxygen-independent organic sources (Vaughan et al, 1998). Both H_2O_2 and Fe (II) are photochemically produced in these sunlit waters. H_2O_2 is formed via the disproportionation of the superoxide, produced by the reduction of oxygen by photo-excited dissolved organic matter (DOM). Fe (II) on the other hand is produced by the photo-reduction of Fe (III), which may be O_2 assisted. The process is usually increased by complexation with organic ligands such as DOM (Fukushima et al, 2001).

Application of the Fenton reaction to environmental systems includes; the remediation of organic matter and inorganic pollutants in waste water and sewage treatment. While several studies have suggested that the HO is the oxidizing species involved in these oxidative processes, other possibilities have not been ruled out. For example, a study of the reduction of dissolved iron species by humic acid has suggested

that in addition to the HO radical, another oxidant may be involved in the Fenton reactions at sea, at neutral pH (7.0- 7.5), (Paciolla et al, 2002). This parallels recent studies (Hug et al, 2003) that have indicated that HO is involved in the oxidation of arsenic (III) to arsenic (IV), which occurs readily at low pH, and that high-valent metal species may be formed at high pH which do not readily oxidize the arsenic (III). Other recent studies (Gonzalez-Davilla et al, 2005), suggest that at nanomolar levels of Fe(II), the oxidation of Fe(II) by H_2O_2 in seawater predominantly involves the $FeOH^+$ species at pH 6 -8.

Determining the nature of the species involved in these reactions, is key to the design of better environmental remediation systems employing the Fenton reaction, and in understanding the chemistry of persistent environmental pollutants such as arsenic (III).

1.6 Methods of Analysis and detection of radical species

Fast, time–resolved methods such as pulse radiolysis (e.g. of water, Reaction 1.21) and flash photolysis (e.g. of H_2O_2, Reaction 1.22) are widely used in order to study the kinetics and mechanisms of the reactions of free radicals. Pulse radiolysis, a method commonly used by radiation chemists, provides "clean" sources of free radicals for study. Normally, the desired radicals are generated in a time much shorter than their reaction time, and the rate of change of their concentration as a function of the solute concentration, is monitored.

$$H_2O \xrightarrow{\gamma\text{- rays}} e^-_{(aq)} + HO^{\bullet} + H^+ \qquad 1.21$$

1.22

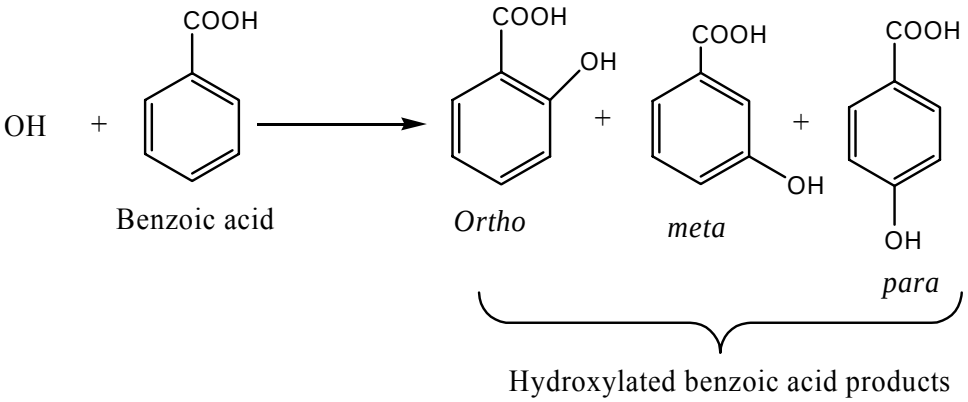
In most of the free radical studies, rate constants of free radicals from pulse radiolysis studies are used to test the characteristics of the species involved in the reactions of interest to determine if they conform to that known for the radical species. A large part of the controversy regarding the nature of the species involved in the Fenton reaction, has therefore emanated from the type of method (usually indirect) used to detect the presence of these species. The species involved are believed to be either so transient in nature or very highly reactive with very short life times that they have steady state concentrations way below the detection limits of most conventional techniques.

It is difficult for example, to demonstrate the involvement of high-valent metal species considering their unavailability (especially at neutral or acidic pH) from an independent source (Eberhardt, 2001). The formation of the ferryl species in Fenton reactions is therefore indirectly deduced from the presence of a species exhibiting reactivities different from the hydroxyl radical (Yamazaki et al, 1991). In most of the studies, the production of HO has been determined by the use of spin traps (e.g. DMPO, Scheme 1.5), scavenger molecules (e.g. alcohols) or product analysis e.g. (GC-MS). Although these techniques have been used widely and may yield expected results, they are subject to several often severe limitations (Table 1.1).

Scavenger molecules such as ethanol, mannitol or formate have been used in competition experiments to infer the formation of hydroxyl radicals (e.g. Reaction 1.23). However, it has been shown that these compounds not only react with hydroxyl radicals

but with other oxidizing species as well (Yamazaki et al, 1991, Czapski, 1984), and may therefore give inconclusive results.

In product analysis, molecules known to react with HO such as benzoic acid may be used. The final products resulting from the reaction of HO with these molecules are determined and compared with those expected for HO reactions. In the case of benzoic acid for example, the final major products are the *ortho*, *meta* and *para* hydroxylated benzoic acid.



The problem associated with this method however is that the resulting oxidation products for some organic substrates such as alcohols may also result from reactions with other oxidizing intermediates the method does not therefore provide unequivocal evidence for the involvement of a particular species.

Electron paramagnetic resonance spectroscopy (EPR) and spin traps (e.g. DMPO, Scheme 1.3) have been used widely to scavenge transient radicals to produce persistent nitroxyl radicals (spin adducts). The EPR signal intensity and pattern of the nitroxyl radicals inform about the magnitude and identity of the trapped radical. This technique, however, is subject to numerous limitations (e.g. case of DMPO, Scheme 1.3) and

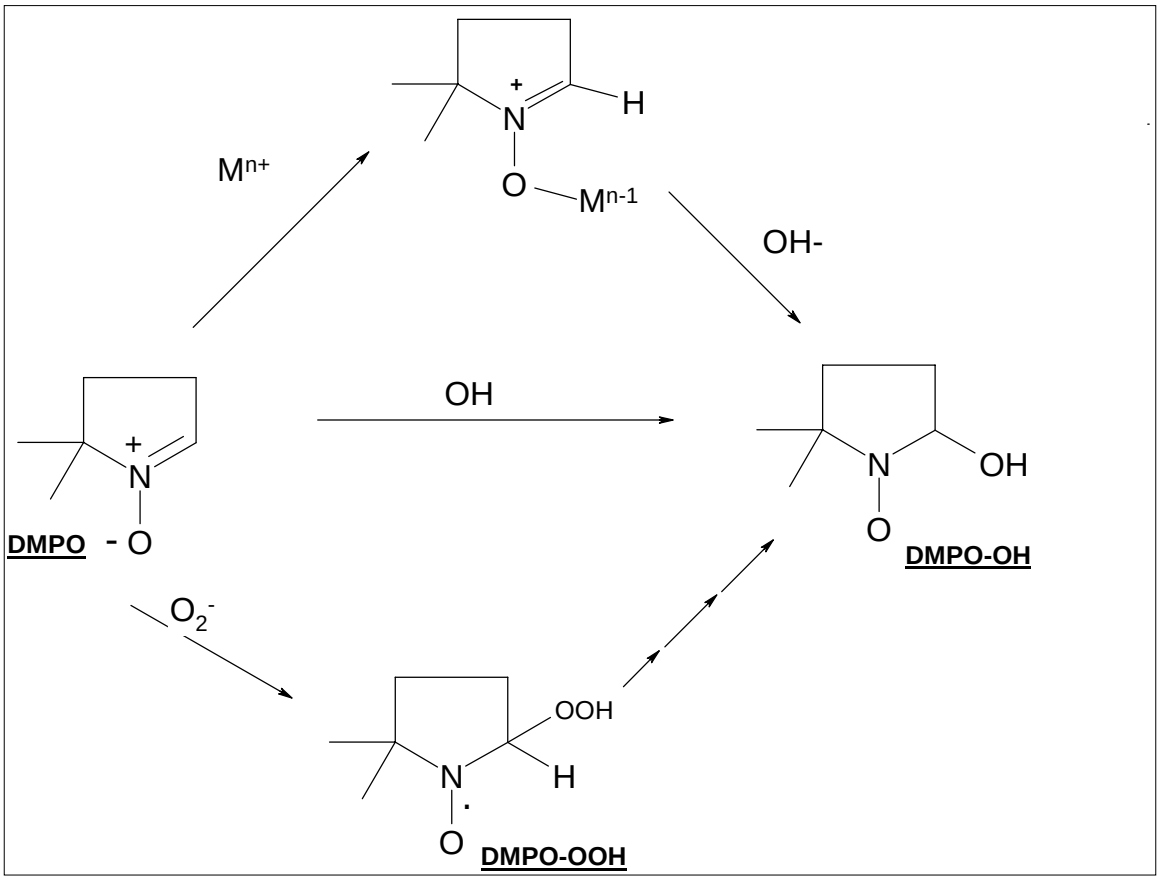
artifacts including: low reactivity, sensitivity, spin adduct instability, artifactual production of spin adducts and the need for high concentrations of spin traps to scavenge radicals because of the low rate constants for the trapping reaction (Filkelstein et al, 1980).

Utilization of these techniques not only limits our understanding of the mechanisms and amount of the reactive oxygen species, but also clouds our ability to identify them in Fenton and Fenton like reactions (FLRS).

Table 1.1 Methods commonly employed in the study of free radicals

Method	Advantages	Disadvantages
Product analysis	Employs well established analytical techniques	Does not always provide unequivocal evidence; may be less discriminate
Inhibition by free radical scavengers	Simple technique	Little information about structure of radical, can be non-specific
Inhibition by superoxide dismutase	Simple and highly specific	Only applicable to superoxide
Direct EPR	Yields structural information	In most cases, radicals are short lived with steady state concentrations below the detection limit of the instrument
Indirect EPR	Wide application	Structural information may be incomplete
Our Approach	Combines several techniques; product analysis, multiple probe molecules and kinetic studies	Systematic approach, requires more effort

Scheme 1.5 Spin trapping with 5, 5, dimethyl Pyrroline-N-oxide (DMPO)



Scheme 1.5 Spin trapping with DMPO. The formation of DMPO-HO may result from direct reaction of DMPO with HO or it may be as a result of oxidation of the DMPO by metal ions in solution. The same product DMPO-HO may also be formed by reaction of DMPO with superoxide through a series of steps in aqueous solution. This scheme typifies the magnitude of artifacts that may result from use of spin traps in the detection of HO. Appearance of the EPR spectra corresponding to DMPO-HO will therefore not be a sure indicator of the presence of HO.

1.7 Specific Aims

Despite these advances, it is evident that the nature of species involved in Fenton and Fenton-like reactions with organic or inorganic ligands is still unclear.

A new highly sensitive technique has been developed by Li et al, (1997) and Kieber and Blough, (1990), for the trace determination of HO , as well as carbon-centered radicals. This technique has been applied successfully in determining rates of hydroxyl radical production in biological systems (Li et al, 1997). The method employs the reaction between HO and dimethyl sulfoxide (DMSO) to produce a methyl radical quantitatively. The methyl radical then reacts with a nitroxide (3ap, I in Scheme 1.5) to yield a methyl-nitroxide product (CH_3-3ap) which is derivatized with fluorescamine to produce the stable *O*-methylhydroxylamine (II, Scheme 1.5). II is then separated by reverse-phase high performance liquid chromatography (HPLC) and quantified fluorometrically.

A modification of this technique is hereby successfully applied to test the nature of species formed in Fenton reactions. In order to achieve this, the Fenton reactions are carried out in presence of a primary scavenger DMSO and in presence of appropriate amounts of the nitroxide. The yield of II in these reactions would be related to the quantity of oxidizing intermediates generated if the oxidizing intermediates are able to react with the primary scavengers such as DMSO to yield methyl radicals. By replacing DMSO with methane, one can determine if the oxidizing intermediates are sufficiently strong to abstract hydrogen from methane to yield methyl radicals. This is because, the HO reaction with methane involves H atom abstraction, not addition as in the case with

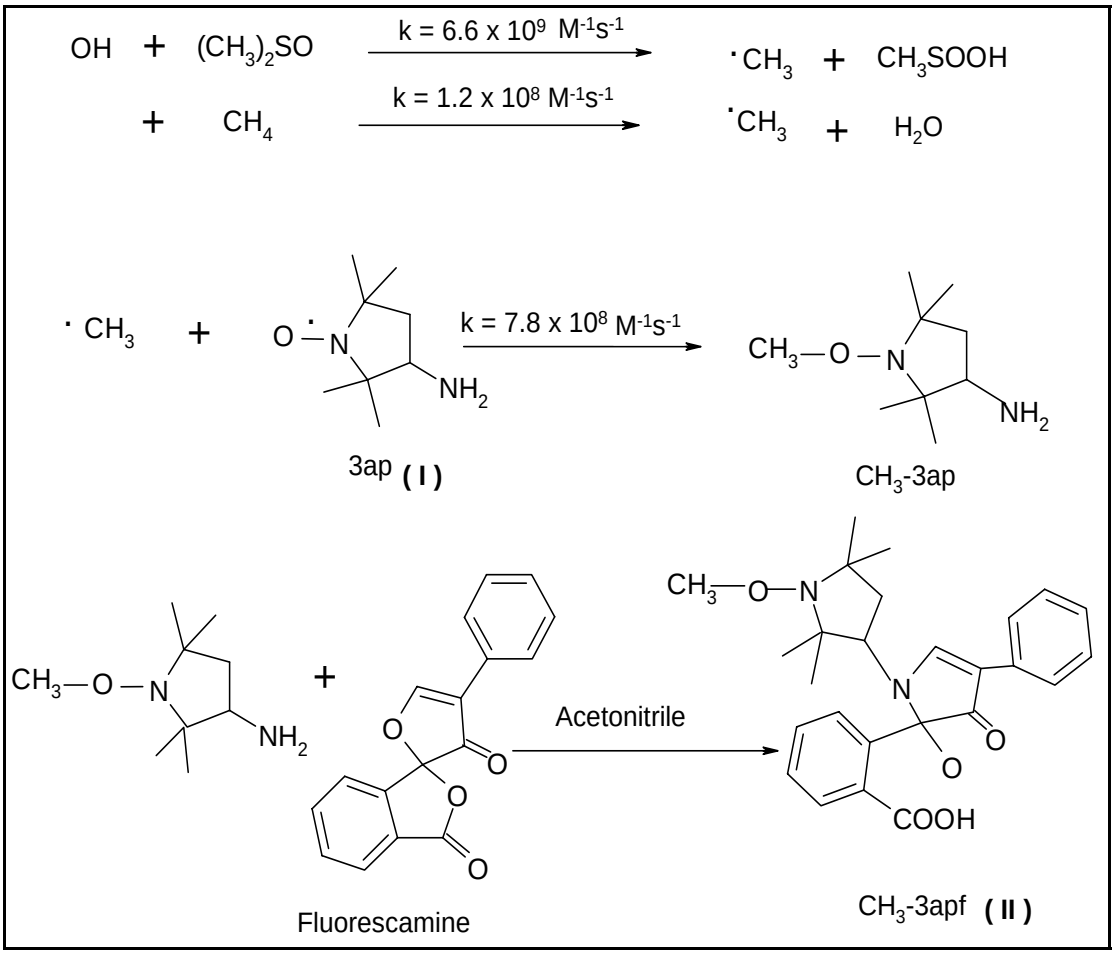
DMSO, and only sufficiently strong oxidizing intermediates may be capable of abstracting a hydrogen atom from it.

Competition experiments will also be performed whereby a second free radical scavenger such as benzoic acid or the nitroxide itself is introduced in addition to the primary scavenger DMSO or methane. In such an instance, the decrease in II yield as the concentration of the competitor is increased significantly can provide further indication of the nature of the species involved. Furthermore, a comparison of II yield in the DMSO and methane experiments carried out under identical (similar), conditions would be performed to test whether the ratio of II yield in the DMSO and methane experiments conforms to those expected for HO.

In order to achieve these goals, Fenton reactions will be carried out in the presence of inorganic ligands i.e. phosphate ligands resulting from the phosphate buffer used (chapter II). These experiments are carried out under acidic (pH 4.2) and neutral (pH 7.4) conditions. In chapter III, the Fenton reactions are carried out with organic ferrous complexes of EDTA, DTPA and NTA. The organic ligand studies are performed more extensively at neutral pH where the ligands are expected to chelate and solubilize the metal ions more strongly than at pH 4.2; however, some studies are also done at lower pH 4.2, for comparison purposes. In chapter IV, key findings of this study are summarized and insights into future research directions pertaining to this work provided.

This study clearly shows that HO is involved in Fenton reactions under all the experimental conditions used. It is further shown here that varying the experimental conditions such as pH or nature of iron complexing ligand significantly influences the

yield of II associated with the quantity of oxidizing intermediates generated, possibly due to competing side reactions.



Scheme 1.6 HO Trapping Method (Kieber & Blough 1990, Li et al 1997)

CHAPTER II

Fenton Reactions with Inorganic Metal Complexes

2.1 Introduction

Considerable debate regarding the nature of intermediates involved in Fenton and Fenton-like reactions still exists (Sawyer et al, 1996, Walling et al 1998, Meyerstein and Goldstein, 1999, MacFaul et al, 1998). While some studies have explicitly stated that Fenton reagents do not produce free $HO^{\cdot}$, free carbon radicals ($R^{\cdot}$) or aryl products (HO-$Ar^{\cdot}$) (Sawyer et al, 1996, Dunfold, 2002), and instead insist on the involvement of metal based oxidants, such assertions have been highly disputed, with studies indicating formation of free radicals in one-electron transfer reactions (Walling et al, 1973, 1974, and 1975, Czapski et al, 1971). Yet others have cautioned against such generalizations and instead pointed out that the nature of oxidizing species involved in Fenton like reactions may largely depend on the reaction conditions (Meyerstein et al, 1999).

A vast majority of Fenton and Fenton-like reactions have been carried out under conditions where the metal is chelated to inorganic ligands, present from the inorganic salts in solution or in most cases from the buffer used (e.g. phosphate). One key aspect is the influence these ligands may exert on the reactivity of the metals with regard to their ability to generate oxidizing intermediates. Many studies with inorganic metal complexes have shown that the oxidizing intermediates generated in the presence of H_2O_2 exhibit

reactivity with organic substrates similar to that of HO (Walling et al, 1973-1975, Czapski et al, 1971, Eberhadt and Colina 1988).

Proponents of metal-based oxidants have pointed out that although HO reacts with methane, ($k = 1.2 \times 10^8\ M^{-1}s^{-1}$), the intermediates produced in Fenton reactions are unreactive towards methane (Sawyer et al, 1996), and that no one has been able to get a Fenton-like reagent to react with methane (Dunfold, 2002). These assertions form the basis of our studies of the Fenton or Fenton-like reactions with methane.

The debate regarding the nature of oxidizing intermediates involved in Fenton reactions is manifested both in acidic and neutral pH. Accordingly, our studies will be performed at both of these pH conditions. At acidic pH, several studies have indicated involvement of high-valent metal intermediates (Bossman, 1988, Pignatello et al, 1999 and Kremer, 1999) and disputed the extensive work of others that favor involvement of HO under these conditions (Walling et al, 1973 and 1975, Czapski et al, 1971).

Due to the fact that ferryl species and other high valent metal species tend to be stabilized at elevated pH, the greater part of the controversy lies in the nature of species involved at neutral pH. The work of Rush and Koppenol, (1987) suggests that at neutral pH, the Fenton reaction is the only source of HO in aqueous solutions containing ferrous or ferric ions and hydrogen peroxide, although this conclusion has since been contested (Kremer, 2000). Studies at alkaline pH 8.5 - 12.5 suggest that Fe (IV) and Fe (V) are able to react just like HO preferably with protonated forms of amino acids leading to chain reactions, contrary to the general belief that such chain reactions are only typical of free radicals (Shepherd et al, 1992). Other studies at neutral pH have suggested that even though HO is exclusively obtained from H_2O_2 it is difficult to distinguish between HO

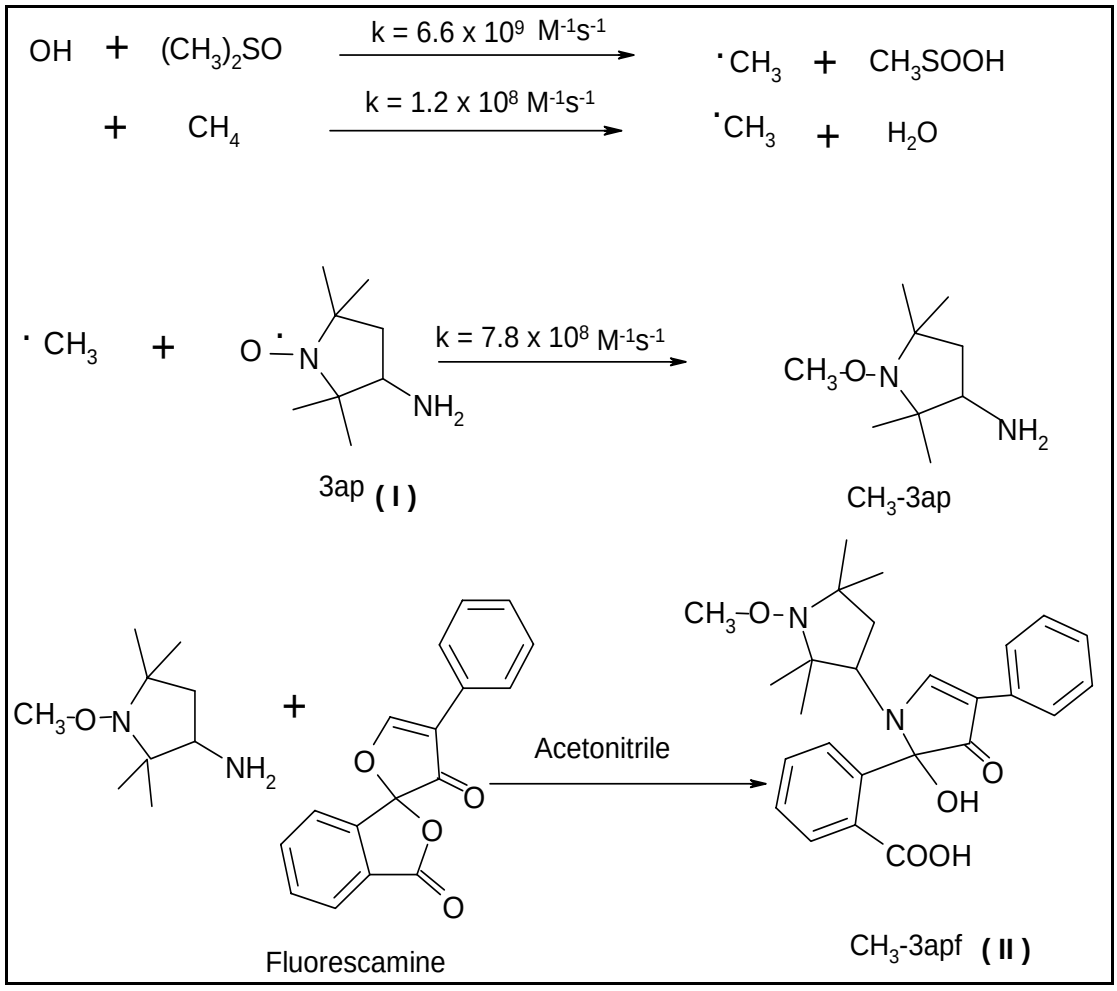
and ferryl species if both are derived from the peroxide (Lloyd et al, 1997). Most studies have favored free radical involvement under these conditions (neutral pH with inorganic metal complexes). Some of these studies suggested that in instances where high-valent metal species are thought to be involved, as is the case where free radical scavengers fail to inhibit reaction, a site specific reaction mechanism is involved. Under this mechanism, the radicals react with molecules near the site of formation before release to solution to react with HO scavengers (Czapski et al, 1983 and Samuni et al, 1983).

The key objectives of the study with inorganic metal complexes is to determine the nature of intermediates involved in Fenton reactions at low and neutral pH and factors that influence their generation. To achieve these objectives, the Fenton reaction was carried out in presence of DMSO and then in the presence of methane and a determination and comparison of II yields (Scheme 1.5) from the two experiments performed. In this way, any intermediates generated by the reaction will be expected to react with the primary scavengers DMSO or methane and to yield methyl radicals which will eventually lead to formation of II.

Competition experiments were performed at increasing concentrations of 3ap as well as benzoic acid and the decrease in yields of II for the two experiments examined and compared with those expected from pulse radiolysis studies for HO. Finally, the Fenton reaction was carried out at increasing metal ion concentrations to give an idea of the stoichiometry of the reaction.

These studies indicate that the intermediates produced in Fenton reaction of inorganic metal complexes are indistinguishable from HO. Hydroxyl radical is produced in Fenton reactions both at low and high pH and the yield of II associated with HO is

found to diminish with increasing pH possibly due to competing side reactions such as oxidation of the metal ions by oxygen.



Scheme 1.6 HO trapping method; (Kieber & Blough 1990, Li et al 1997)

2.2 Experimental

2.2.1 Apparatus

All absorption spectra were measured with a Hewlett Packard 8452A diode array spectrophotometer. Monochromatic irradiations were performed using a 1000-W Hg-Xe arc lamp powered by a Spectral Energy 256SM power supply after passage through a GM 252 monochromator set to a band pass of 10 nm. The lamp irradiance was measured using an International Light IL 1700 radiometer.

The HPLC system consisted of an Eldex B-100S single piston pump (Eldex Scientific CA, USA) which was equipped with a gradient controller (Elab, supplied by OMS Tech Florida, USA). The connection line included a (0-5000) psi liquid pressure gauge, two pre-column filters and a Valco C-10W injection valve (Valco Inc., Texas, and USA). A Waters C-18, 4-μM reverse-phase Nova-pak cartridge column housed in an RCM 8x10 cm radial compression module was used (Waters, Massachusetts, USA), and a loop size of 50 μL was employed for all measurements. An Applied Biosytems UV detector 785A (Bodman, USA), and Hitachi L-7480 fluorescence detector (Hitachi Scientific Instruments, Japan), were connected in series for absorbance and fluorescence detection respectively. Data acquisition and analysis was done on computer using Elab software (OMS Tech, Florida, USA). Chromatographic separations were performed at 27^{o}C employing isocratic elution with mobile phase composition of 65% MeOH and 35% acetate buffer at pH 4.0, at a flow rate of 1 mL/min.

2.2.2 Materials

Sodium hydrogen phosphate, (Na_2HPO_4), sodium dihydrogen phosphate, (NaH_2PO_4), (99.999%), dimethyl sulfoxide, (DMSO 99.9%), sodium hydroxide pellets, (NaOH 99.998%), ferrous sulphate pentahydrate, ($FeSO4.7H_2O$), acetonitrile, (ACN 99.93 %), boric acid (99.999 %) and fluorescamine were all obtained from Aldrich or Sigma Aldrich. 3-amino-2, 2, 5, 5, tetramethyl-1-pyrolidinyloxy free radical (3ap I, 99 %) was obtained from Across and iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) obtained from Across Organics. Hydrochloric acid (HCl 36.5-38%), glacial acetic acid and HPLC grade methanol were obtained from J.T. Baker, whereas, hydrogen peroxide (H_2O_2, 30 %) was obtained from Fisher. Ultra High Pure (UHP) grade nitrogen and methane gases were obtained from Airgas, Inc. Radon, PA, whereas gas-tight syringes with sizes ranging from 10 µL to 5mL were obtained from Hamilton Inc. USA. 0.2 µm nylon membrane filters were obtained from Whatman, England.

2.2.3 Experimental Preparations

Phosphate buffer was prepared from high purity (99.9999%) salts by first preparing 1.0 M solutions of the monobasic and dibasic salts and mixing appropriate volumes to achieve 100 mM phosphate buffer at the desired pH. Lower concentrations of the buffer (e.g. 2 mM) were made by appropriate dilutions using distilled, de-ionized water and adjusting the pH to that desired using dilute HCl or NaOH solutions. All the buffer solutions were stored at 4^oC in the dark. Acetate buffer was prepared by adding 3-mL of

glacial acetic acid into 1L of distilled deionized water. The resulting mixture was then stirred thoroughly for 5 minutes under the hood before adjusting the pH to 4.0 with sodium hydroxide. The buffer solution was then filtered through 0.2 μm nylon membrane filters with vacuum filtration apparatus and stored in dark bottles on the bench. Borate buffer of desired concentration (e.g. 200 mM or 400 mM) was prepared from high purity boric acid in distilled deionized water by adding about 4 pellets of high purity NaOH, and then adjusting the pH (between 8.0 and 9.0) using dilute solutions of NaOH or HCl.

Stock solutions of 0.1 or 0.2 M dimethylsulfoxide (DMSO) were prepared from the high purity reagent, with lower concentrations obtained from these by dilution. Eight mM stock solutions of fluorescamine in acetonitrile were prepared daily and stored in the dark. Stock solutions of 3ap were prepared monthly in either distilled deionized water or 2 mM phosphate buffer of the desired pH. The stock solutions were then filtered through 0.2 μM syringe filters and stored at 4 ^{0}C in the dark. The concentration of the 3ap used was determined daily by absorption spectrophotometry using an extinction coefficient of 2200 $M^{-1}cm^{-1}$ at a wavelength of 234 nm obtained from 3-(carbamoyl)-2,2,5,5, tetramethyl-1-pyrrolidinyloxy standards.

Stock solutions of 5.0 mM ferrous sulfate were prepared daily by dissolving a weighed amount of the salt into nitrogen-purged 2.0 mM phosphate buffer at pH 4.2. The resulting solution was vortexed and N_2 purged for 30 minutes to ensure proper mixing. Lower concentrations of the ferrous salt (400 μM) were then prepared from the stock solution by transferring appropriate volumes of the stock using a gas-tight syringe into 2 mM phosphate buffer under nitrogen. The resulting solution was purged continuously with nitrogen throughout the day. The ferrous solutions were prepared in 2-mL

borosilicate micro vials having a Teflon seal at the top; nitrogen was delivered to the micro-vials by stainless steel pipes and a needle inserted into the vial through a hole at the top, with a second needle inserted at the top for effective purging. 200 mM ferric sulfate solutions were prepared daily or weekly in water or phosphate buffer and stored at $4^{o}C$ in the dark.

5.0 mM hydrogen peroxide stock solutions were prepared daily and their concentrations determined spectrophotometrically using a molar extinction coefficient of 56.4 M^{-1} cm^{-1} at a wavelength of 240 nm. HPLC mobile phase, which consisted of 65 % MeOH and 35 % acetate buffer pH 4.0, was prepared by mixing appropriate volumes of the acetate buffer and methanol.

2.2.4 Experimental Protocol

2.2.4.1 Product Formation; Fenton reaction in presence of DMSO

To determine whether the oxidizing intermediate(s) involved in the Fenton reaction were capable of oxidizing DMSO, the Fenton reaction was carried out in presence of this primary radical scavenger (DMSO), in a cuvete with total reaction volume of 4 mL. Initially, a given volume of 100 mM phosphate buffer at pH 4.2 or 7.4, excess DMSO (minimally, 1.5 mM), and 50 μM 3ap were added to the cuvette and the solutions purged with nitrogen gas for 5 minutes. A gas-tight syringe was used to transfer 150 μM hydrogen peroxide to the cuvette from a stock solution that had been previously purged with nitrogen for 15 minutes. The reaction was then initiated by addition of 10

μM Fe (II) via a gas-tight syringe from the 400 μM ferrous stock solution. The reaction was then allowed to proceed to completion (~15 minutes).

An aliquot of the product was then drawn and its pH raised to above 8.0 by addition of 400 mM borate buffer at pH 9.0. The nitroxide in this sample was derivatized by adding 8 mM fluorescamine solution to ensure at least a 5:1 ratio of fluorescamine to nitroxide in the derivatized mixture. The derivatized sample was quickly shaken and the reaction allowed to continue in the dark for at least a minute prior to injection into the HPLC, where II was detected and quantified. UV detection of II was done at a wavelength of 390 nm and fluorescence detection was done at an excitation wavelength of 390 nm and emission wavelength of 490 nm.

2.2.4.2 Fenton reaction in presence of methane

As a second test of the nature of oxidizing intermediate(s), the Fenton reaction was performed in presence of methane in place of DMSO. The reaction was carried out in a teflon-sealed quartz cuvette with a total reaction volume of 4 mL. In this case, a given volume of 100 mM phosphate buffer at pH 4.2 or 7.4, and 50 μM 3ap were added to a sealed cuvette, which was then purged for at least 10 minutes (saturating methane) with UHP methane gas delivered by stainless steel tubes and needles through the top of the cuvette.

In order to ensure saturating methane (i.e. dissolution of CH_4 in water to its maximum solubility concentration of 1.5 mM), the Fenton reaction experiments were carried out under methane (purging) for different time periods, (i.e. 1, 3, 5, 10 and 30 minutes) and it was found that yield of II was constant after 5 minutes of CH_4 purging.

However, to ensure uniformity, the reaction mixture containing the buffer and 3ap solution were CH_4 purged for 10 minutes before addition of the peroxide and Fe (II) ions to initiate the reaction, which was further performed under CH_4 gas.

N_2 purged H_2O_2 (150 μM) was then added via gas-tight syringe to the reaction mixture with continual purging. The reaction was initiated by addition of 10 μM ferrous ions delivered by a gas-tight syringe from a 400 μM Fe (II) stock solution under nitrogen. The reaction was allowed to go to completion under methane for 15 minutes; a sample was then drawn for the derivatization, separation and quantification steps as outlined in the above section (2.2.4.1).

2.2.4.3 Competition experiments with nitroxide

As a further determination of the nature of intermediates involved in the Fenton reaction, competition experiments were performed using the nitroxide itself as a competitor. Experimentally, the concentration of the nitroxide was increased at a constant DMSO concentration and the product yield monitored. In this case, appropriate concentrations of 3ap (varying between 0.15 mM to 2.4 mM) for each experiment were added to 50 μM DMSO in 100 mM phosphate buffer at pH 4.2. 150 μM hydrogen peroxide with 10 μM Fe (II) was then added to make a total reaction volume of 4.0 mL. The reaction was allowed to continue to completion before derivatization, separation and quantification as discussed above.

2.2.4.4 Nitrate Experiments

Photochemistry of nitrate is a well-known reaction for the production and generation of HO. To establish the rate constant for the reaction of HO with the nitroxide, this known source of HO was employed in competition experiments similar to the one discussed above. Stock solutions of potassium nitrate (KNO_3) were prepared weekly in water or buffer and kept in the dark at room temperature; 500 μM potassium nitrate was then added to a mixture of 50 μM DMSO and 50 μM 3ap in 100 mM phosphate buffer at pH 7.4. The mixture was then nitrogen purged for 5 minutes before irradiation with monochromatic light at wavelength of 315 nm for 30 minutes under continuous purging with nitrogen gas. For the competition study, the concentration of 3ap was varied between (0.025 mM to 1.5 mM) under a constant concentration of the primary trap DMSO. The competition experiment was performed under a range of DMSO concentrations, ranging from 50μM - 100 μM DMSO.

2.2.4.5 Benzoic Analysis Experiments

A purified stock solution (10 mM) of benzoic acid was obtained by recrystallization and preparation in distilled, deionized water. Benzoic acid of appropriate concentration was then added to a reaction mixture containing 50 μM DMSO and 50 μM 3ap in 100 mM phosphate buffer. The resulting mixture was purged with nitrogen for 10 minutes before adding hydrogen peroxide and initiating the reaction with the Fe (II). The reactions were allowed to continue under nitrogen for 15 minutes before separation and quantification using the HPLC system.

The resulting product was analyzed for II formation and for the formation of 2-hydroxylated benzoic acid product. In order to detect the 2-hydroxybenzoic acid, the

fluorescence detector was set to excitation and emission wavelengths of 305 and 410 nm respectively, and a mobile phase composition of 35 % MeOH and 65 % phosphate buffer at pH 2.0 used.

2.2.4.6 Metal ion concentration dependence

The dependence of yield of II on Fe concentration was examined by studies in which Fe (II) concentrations, ranging from 5 μM to 25 μM, were added to a cuvette containing 10 mM DMSO, 50 μM 3ap and 150 μM hydrogen peroxide, to a total reaction volume of 4 mL, under anaerobic conditions as described above in section 2.2.4.1 above. The reaction was allowed to proceed to completion prior derivatization as outlined in section 2.2.4.1 above.

2.3.1.7 Experiments with Fe (III)

These experiments were performed using both DMSO and methane as primary HO scavengers, the purpose being to determine whether the intermediates formed by the reaction of HO with Fe (III) are capable of oxidizing DMSO or methane.

In the DMSO experiments, 50 μM 3ap was added to 100 μM DMSO in 2 mM phosphate buffer at pH 4.2 in a cuvette with a Teflon seal. The mixture was then N_2 purged for 5 minutes before 150 μM H_2O_2 and 10 mM Fe (III) were added via a gas-tight syringe. The reaction was then initiated by immediate addition of 10 μM Fe (II) and allowed to proceed to completion (15 minutes). An aliquot of the sample was then drawn and its pH raised before it was derivatized and then injected onto the HPLC as described

above. The standard reaction (Fe (II)/H_2O_2, without added Fe (III)) was also performed in presence of DMSO under identical conditions for comparison purposes.

In order to test whether presence of large amounts of Fe (III) might affect the sample derivatization reaction, the standard reactions were performed (without Fe III) and two aliquots were drawn for analysis at the end of the reaction. The pH of the first aliquot was raised with borate buffer before it was derivatized and injected onto the HPLC. To the second aliquot, 10 mM Fe (III) was added prior to pH adjustment, derivatization and injection onto the HPLC.

The methane experiments were done by purging a solution of 50 μM 3ap in 2 mM borate buffer at pH 4.2, with methane for 5 minutes before addition of 150 μM H_2O_2 and 10 mM Fe (III) using a gas-tight syringe. This was followed by immediate addition of 10 μM Fe (II) and the reaction allowed to proceed to completion under methane (15 minutes). The standard reaction (Fe (II)/H_2O_2) was also carried out in methane under identical conditions but without Fe (III) for comparison purposes. Similarly, blank experiments with Fe (III) in presence of methane but without Fe (II), were also performed.

2.2.4.7 Preparation of II and Calibration of HPLC

II was synthesized using the method described previously (Li et al 1997, 1998). 3 mM of 3ap, 3 mM DMSO and H_2O_2 were mixed together in 100 mM phosphate buffer at pH 7.5. 3 mM Fe-EDTA was added to the reaction mixture and the reaction allowed to proceed anaerobically in the dark for at least 30 minutes. The reaction sample was then derivatized with fluorescamine at high pH using borate buffer as discussed above. The

derivatized sample was then extracted (solid-phase) using Waters C_{18} Sep-Pak. The Sep-Pak was first rinsed with MilliQ water and methanol prior to loading with the reaction product, which was concentrated as a yellow band. The yellow band was then eluted with a small volume of methanol or acetonitrile which was further reduced by flushing with dry nitrogen.

The yellow solid was then dissolved into the HPLC mobile phase (65 % methanol and 35 % acetate buffer) pH 4.0 and injected into the HPLC. The analyte was then collected directly from the HPLC and extracted into 2 mL of chloroform, which was then evaporated yielding a bright yellow concentrate of II that was stored in the dark -20 ^{o}C. For HPLC calibration, the concentration of the standard II was determined spectrophotometrically, (ε_{386} = 5225 $M^{-1}cm^{-1}$) and several calibration standards obtained from it by serial dilution. These were run on the HPLC, yielding a linear response, and they were often used to test the elution of reaction product II.

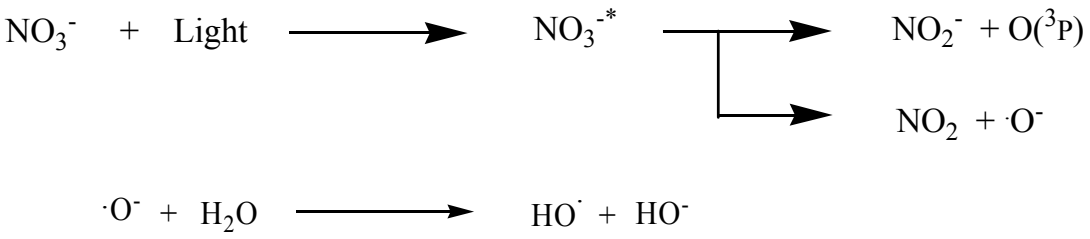
2.3 Results and Discussion

2.3.1 Experiments at acidic pH

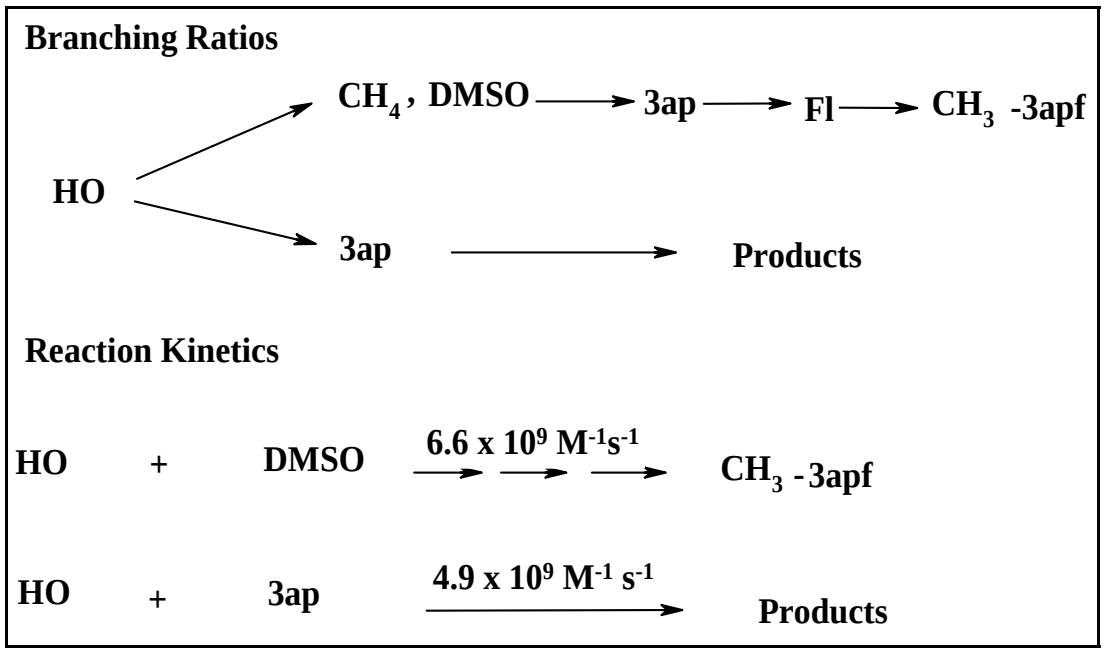
2.3.1.1 Nitrate photolysis experiments

To establish the rate constant for the reaction of HO with 3ap, HO was generated by the photolysis of nitrate. The dependence of the yield of II on 3ap concentration when the nitrate salt was irradiated at a constant wavelength in the presence of fixed concentrations of DMSO was determined.

Irradiation of potassium nitrate at wavelengths above 300 nm produces HO (Narayanswamy L.K 1935).



The generated HO is then subjected to competition reactions with DMSO and 3ap (Scheme 2.1 below).



Scheme 2.1 Expected branching ratios for the competition reaction involving the nitroxide (3ap) at fixed concentration of the primary scavenger (DMSO). The rate constant for the reaction of HO with 3ap was measured experimentally using nitrate photolysis (this study), whereas that of DMSO was obtained from literature (Table B2, Appendix B)

When the concentration of nitroxide is sufficiently high so as to compete effectively with the other scavengers, one can develop a relationship between II yield and the concentration of 3ap at a fixed DMSO concentration.

II formation via the DMSO route will be given by,

$$\mathbf{Y_{DMSO}} = \frac{\mathbf{Y_0\ (k_{DMSO}[DMSO])}}{\mathbf{(k_{DMSO}[DMSO] + k_{3ap}[3ap])}} \qquad \text{II.1}$$

where Y_{DMSO} is the II yield at a given [3ap], and Y_o is the product yield associated with the total HO generated. Similarly, k_{DMSO}, k_{3ap} and k_{CH4} are the rate constants for the reaction of HO with DMSO, 3ap and methane.

This simplifies further to;

$$\mathbf{Y_{DMSO}} = \frac{\mathbf{Y_0}}{\mathbf{1} + \frac{\mathbf{k_{3ap}[3ap]}}{\mathbf{k_{DMSO}[DMSO]}}} \qquad \text{II-2}$$

This expression is in the form,

$$\mathbf{Y_{DMSO}} = \frac{\mathbf{Y_0}}{\mathbf{1} + \mathbf{b}\ \mathbf{[3ap]}} \qquad \text{II.3}$$

where,

$$\mathbf{b} = \frac{\mathbf{k_{3ap}}}{\mathbf{k_{DMSO}[DMSO]}} \qquad \text{II.4}$$

and, the ratio of the rate constants,

$$R_{\frac{3ap}{DMSO}} = \frac{k_{3ap}}{k_{DMSO}} = b\,[DMSO] \qquad \text{II.5}$$

It is therefore expected that a plot of the product formation via the DMSO route with increasing concentration of the nitroxide should give a non-linear regression fit b that corresponds to the expected literature value obtained from the known rate constants of the reaction of HO with 3ap and DMSO and can be used to evaluate the value of the rate constant of 3ap with HO, i.e.

$$k_{3ap} = b(k_{DMSO}[DMSO]) \qquad \text{II.6}$$

The absorption spectrum of nitrate is shown in figure 2.1. Irradiation of nitrate in presence of DMSO and 3ap produced II at levels well above the background (Figure 2.2). When the nitrate irradiation was performed with sufficiently high concentrations of the nitroxide so as to compete effectively with the primary HO scavenger DMSO, a reduction in II yield was observed (Figure 2.3). These experiments done at constant DMSO concentration were repeated several times to assess the accuracy of the rate constant thus determined.

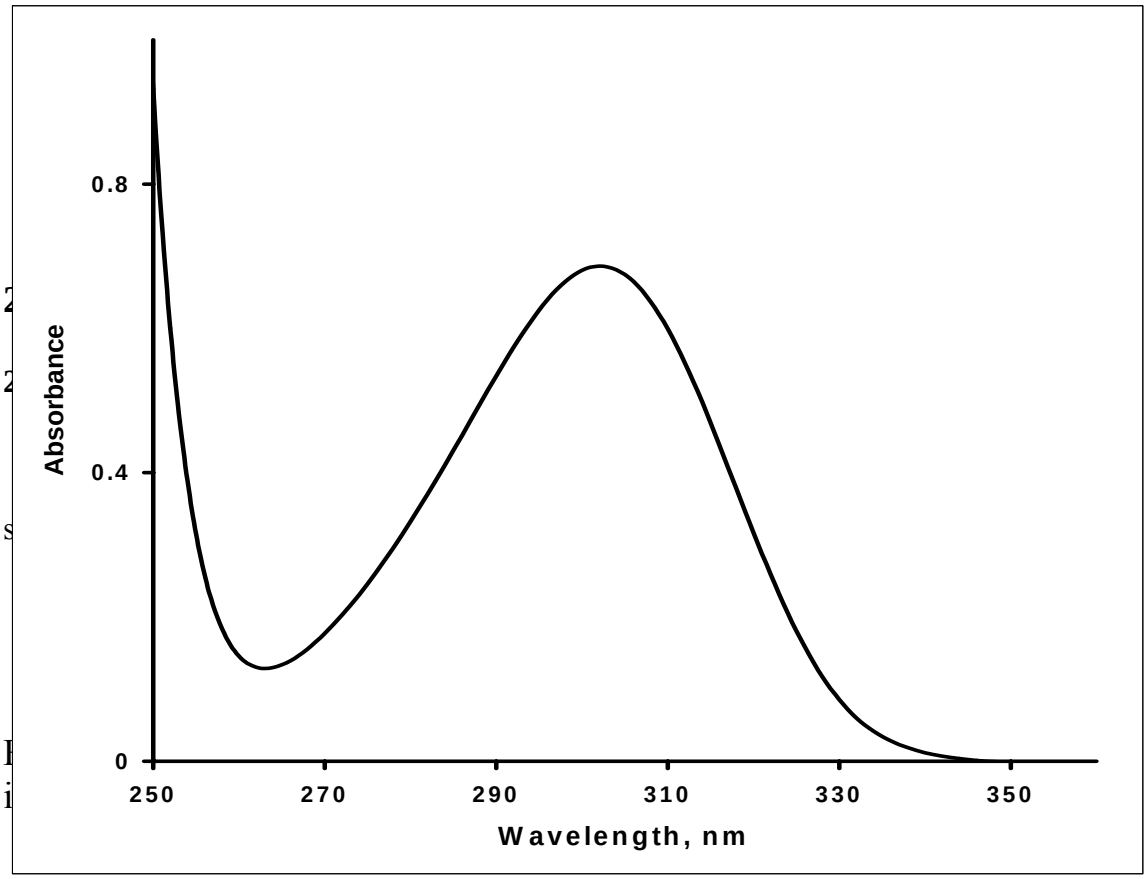


Figure 2.1 UV-VIS spectrum of KNO_3 in water. In the study, the NO_3^- was irradiated with monochromatic light at wavelength of 315nm, with a path length of 1cm.

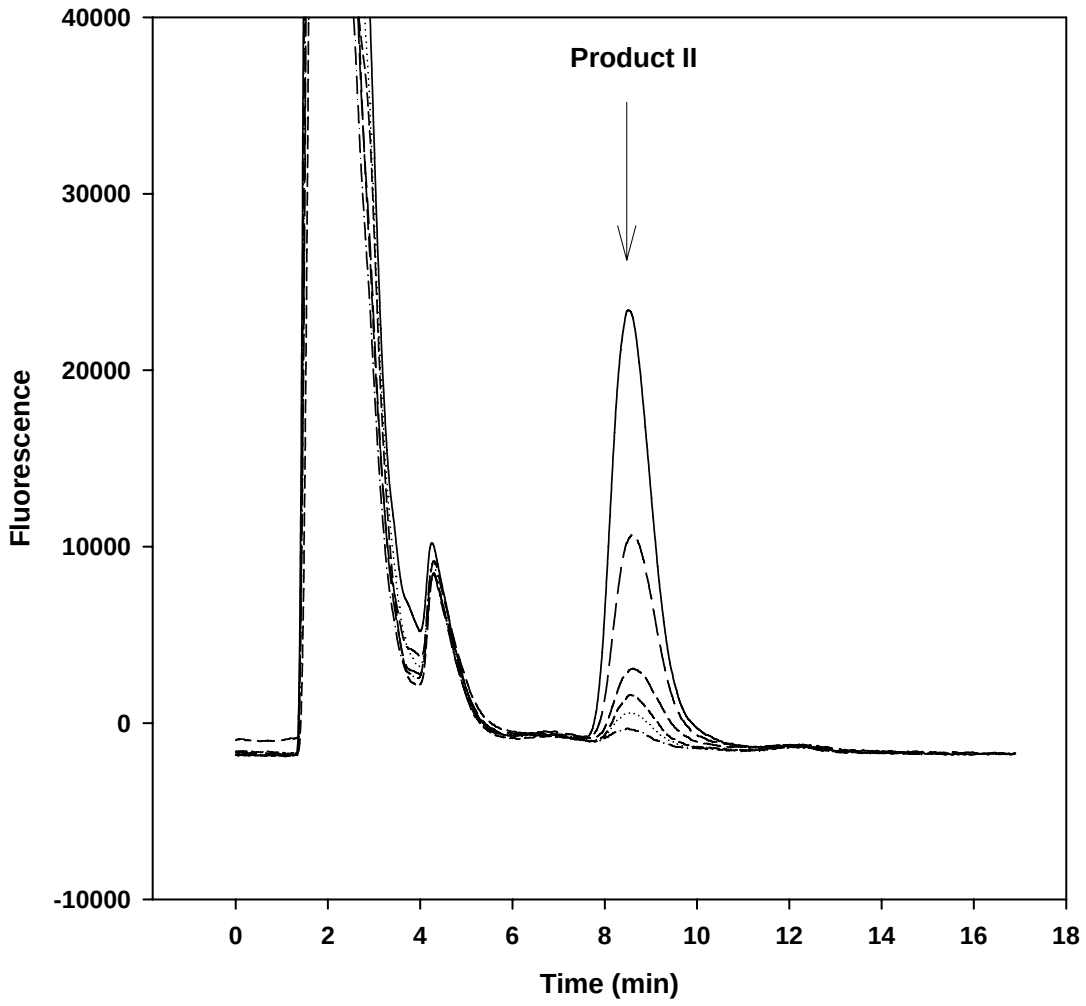


Figure 2.2 Chromatograms illustrating II formation during irradiation of potassium nitrate. Reaction conditions; [3ap] = 50 μM; [DMSO] = 30 μM; [KNO_3] = 500 μM; in 100 mM phosphate buffer at pH 7.4. The reactions were done anaerobically and both standard and blank reactions were followed for 60 minutes, with samples drawn at appropriate intervals for analysis. As shown, nitrate solution irradiated for (——) 60 minutes, (—— ——) 30 minutes, and (— — —) 10 minutes. Whereas blank solution (without KNO_3) irradiated for (-----) 60 minutes (.....) 30 minutes and (— · — · —) 10 minutes.

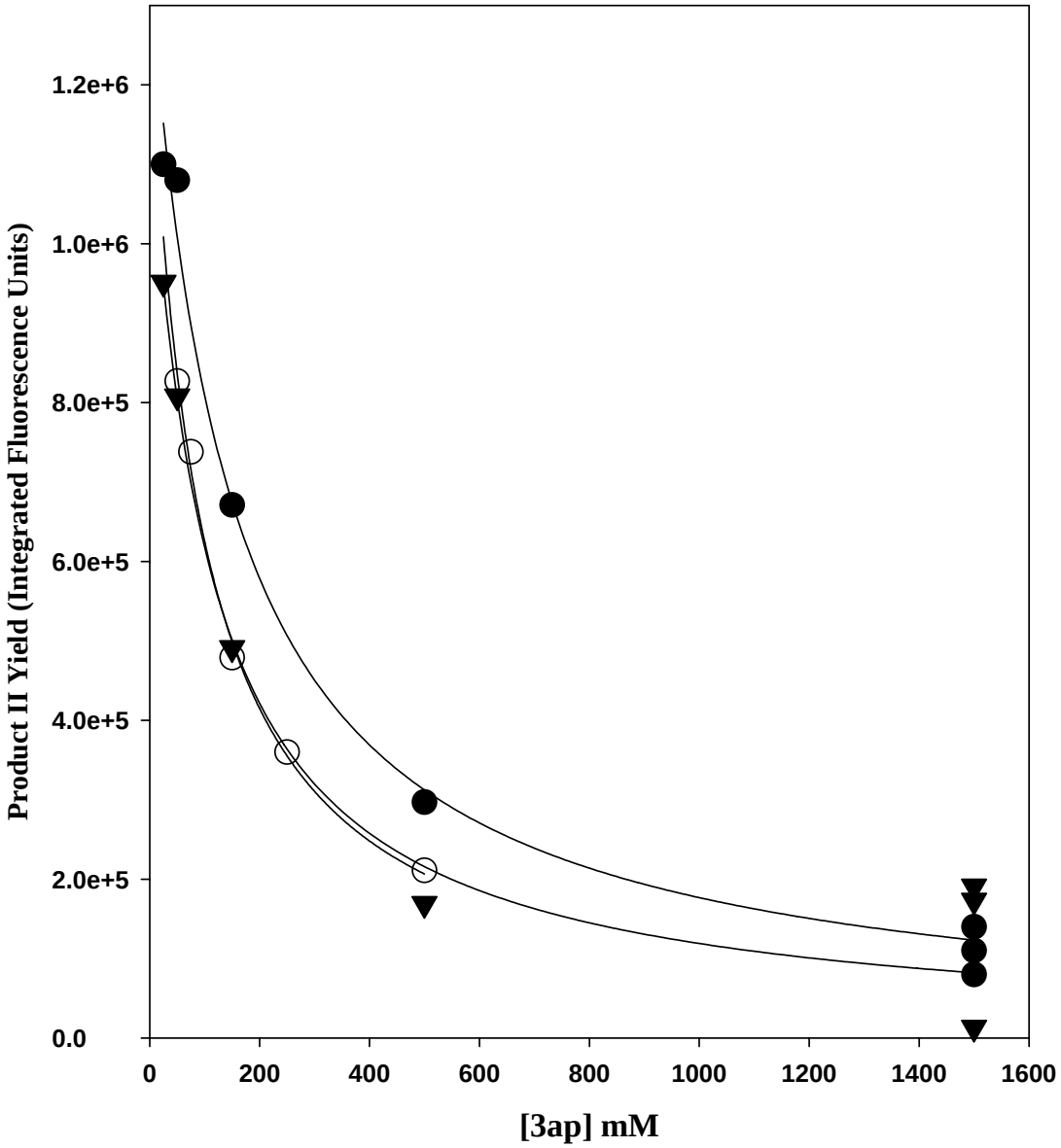


Figure 2.3 Successive decrease in II yield with increasing concentration of 3ap, during irradiation of potassium nitrate under anaerobic conditions with monochromatic light (wavelength 315 nm), in the presence of DMSO at concentrations of (▼) 50 μM, (○) 75 μM, and (●) 100 μM DMSO. The concentration of potassium nitrate was 500 μM and irradiation done in 100 mM phosphate buffer at pH 7.4. The curves represent a non-linear least squares fit to the form $f_n = a/1+ bx$ (see text).

The average value of $k_{3ap} = 4.9 \pm 0.59 \times 10^9\ M^{-1}s^{-1}$ obtained experimentally, compares well with the literature value of $3.7 \times 10^9\ M^{-1}s^{-1}$ (Asmus et al 1976), for the reactions of HO with a similar nitroxide, 3-(carboxy)- 2,2,5,5, tetramethyl-1-pyrrolidinyloxy (3-cp). Furthermore,

Table 2.1 List of findings for the nitrate photolysis study

[DMSO]	$\mathbf{k_{3ap}(M^{-1}s^{-1})}$	$\mathbf{k_{3ap}/k_{DMSO} = b[DMSO]}$
100 µM	4.22×10^9	0.64 ± 0.10
75 µM	5.15×10^9	0.78 ± 0.08
50 µM	5.31×10^9	0.804 ± 0.14
Average	$(4.89 \pm 0.59) \times 10^9$	0.74 ± 0.09

the average ratio of the rate constants k_{3ap}/k_{dmso} obtained experimentally, 0.74 ± 0.09, is close to the expected literature value of 0.56 determined from the known rate constants of the reaction of HO with 3cp (above) and DMSO (Table B2, Appendix).

2.3.1.2 Fenton Reaction: DMSO Experiments

The objective of this experiment was to determine whether the oxidizing intermediate(s) generated in the Fenton reaction are capable of oxidizing DMSO as in the case of the nitrate experiment above, where HO is known to be formed.

When the Fenton reaction was carried out in the presence of DMSO and nitroxide, a significantly high yield of II was obtained (Figure 2.4), which was not formed in the absence of DMSO or metal ions. It was therefore inferred that an oxidizing intermediate capable of oxidizing DMSO to yield methyl radicals and II, just as was found to be in the case of HO in the nitrate experiment, was involved in this reaction.

As a further test of the nature of intermediates produced in this reaction, a competition experiment with 3ap, similar to the nitrate experiment was performed. With increasing concentration of 3ap at a fixed DMSO concentration, the yield of II was found to decrease accordingly. The decrease in yield of II was fit to expression (II.1) used in the case of the nitrate experiment (Figure 2.2), and gave an experimental ratio of the rate constants $k_{3ap}/k_{DMSO} = 1.2 \pm 0.5$ which compared well with the ratio of $k_{3ap}/k_{DMSO} = 0.74 \pm 0.09$ obtained in the nitrate experiment, providing very strong indication that HO is involved in this reaction.

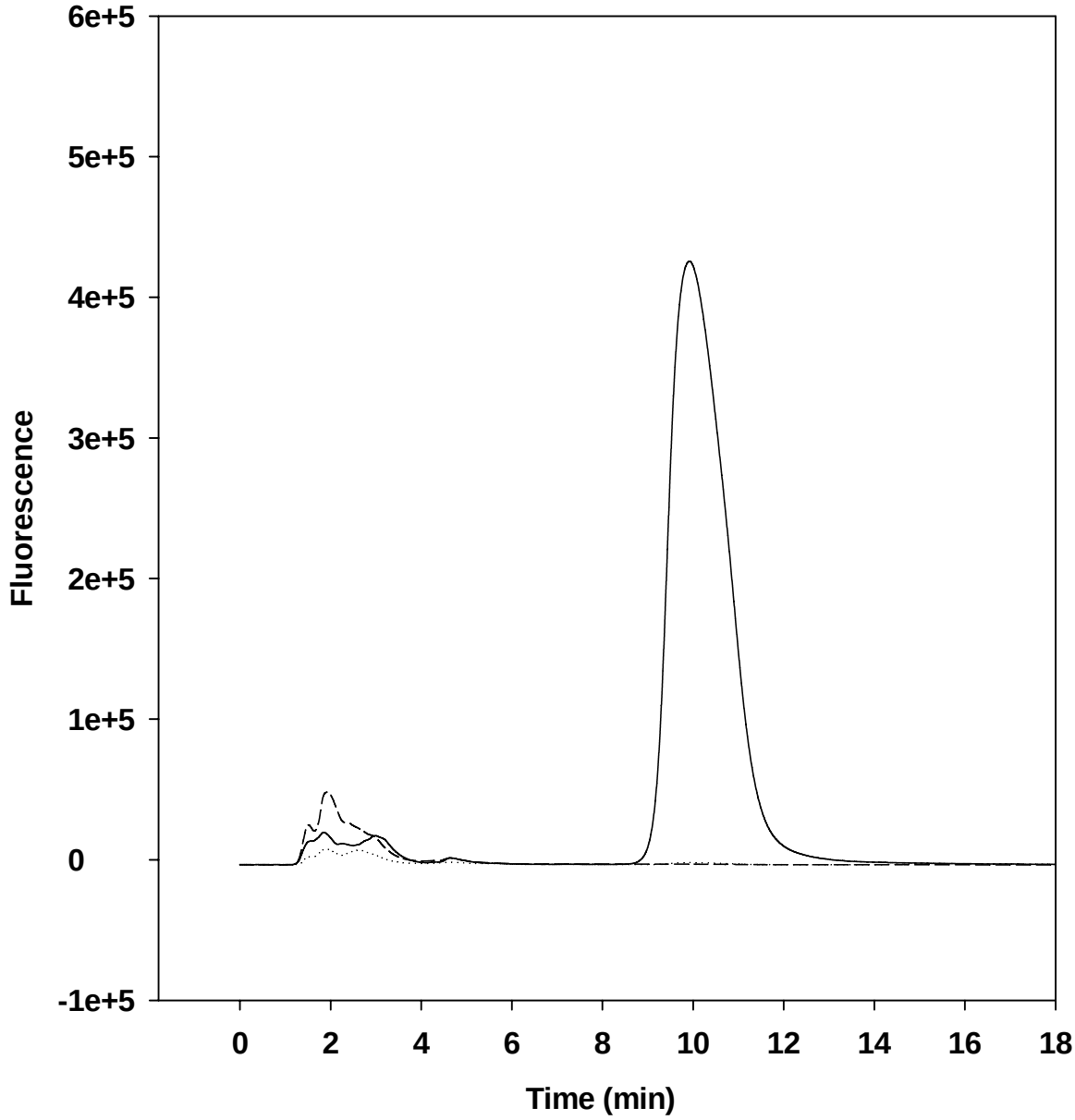


Figure 2.4 Chromatograms illustrating II formation in the Fenton reaction in the presence of DMSO and 3ap under anaerobic conditions. Reaction conditions; [3ap] = 50 μM; [DMSO] = 10 mM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) and allowed to continue for 15 minutes. In standard reaction (——), standard reaction without Fe (II) (– – –), and standard reaction without DMSO (.....). The maximum yield at 15 minutes corresponds to 9.3 μM II.

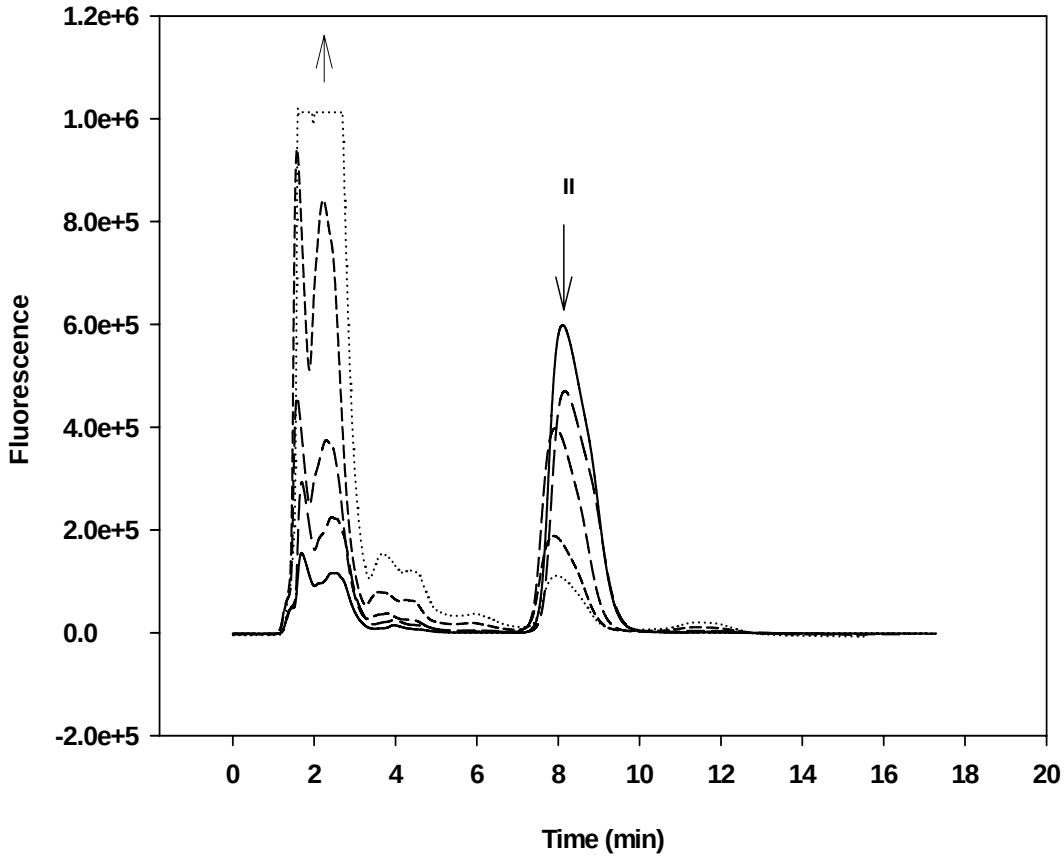


Figure 2.5 Chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. Reaction conditions; [DMSO] = 500 μM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of 3ap; (——) 0.15 mM, (– – –) 0.30 mM, (—— ——) 0.60 mM, (-----) 1.2 mM, and (.....) 2.4 mM respectively. The arrow at ~9 minutes indicates decrease in product II yield with increasing concentration of nitroxide. The arrow at ~ 3 minutes represent increase in the hydroxylamine product formed from excess nitroxide.

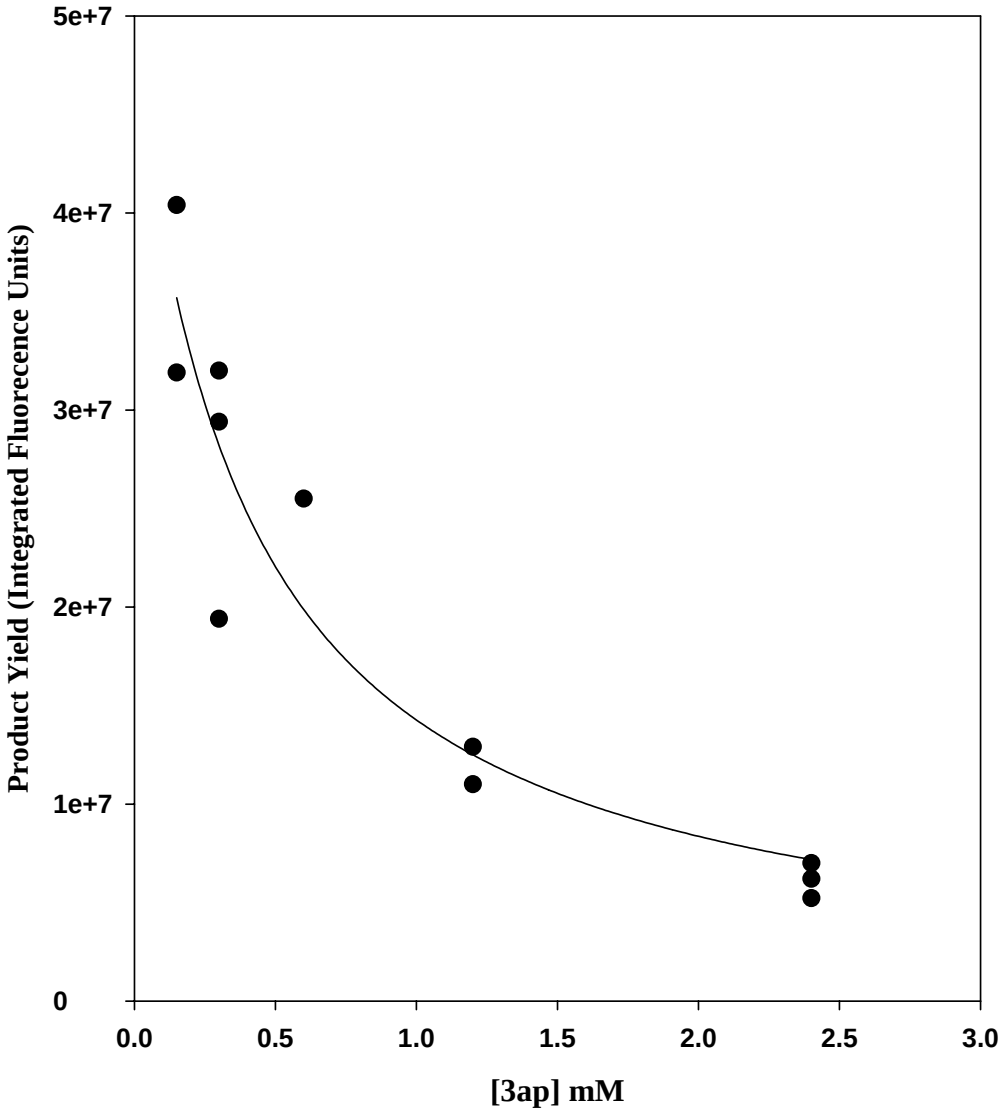


Figure 2.6. The dependence of II yield on [3ap] in presence of a fixed concentration of DMSO. Reaction conditions; 500 μM DMSO, 10 μM Fe (II), 150 μM H_2O_2, in 100 mM phosphate buffer at pH 4.2. The reaction was initiated by addition of the Fe (II) and allowed to continue anaerobically for 15 minutes. The curve represents a non-linear regression of the form $f_n = a/ 1 + bx$. The maximum yield of II was found to be 6.9 ± 1.1μM, when the concentration of 3ap was 50 μM.

2.3.1.3 Fenton Reaction: Methane Experiments

As a further test of the nature of oxidizing intermediate(s) involved, Fenton reactions were carried out using methane in place of DMSO. Since methane, unlike DMSO, has four strong sp3-hybridized C-H bonds, only sufficiently strong oxidizing intermediates may be able to abstract a hydrogen atom from it. Moreover, these experiments directly addressed the issues raised by both Sawyer et al 1996 and Dunfold 2002 regarding the inability of Fenton and Fenton-like reagents to oxidize methane as would be expected if the species were HO. It is therefore expected that formation of II in presence of methane would be an indication that the species involved in these reactions is HO.

When the Fenton reaction was performed in presence of methane and 3ap, significant formation of II was observed, (Figure 2.7), which was not observed in the absence of either methane or metal ions. This observation indicated that a sufficiently strong oxidizing intermediate was involved in the Fenton reactions under these conditions, capable of oxidizing methane to yield methyl radicals and resulting in formation of II. This study shows for the first time that a Fenton reagent is capable of reacting with methane to yield methyl radicals.

By comparing the ratio of II yields for Fenton reactions performed in the presence of DMSO and methane otherwise under identical experimental conditions, we can test whether the ratio (of II yields) for the two experiments is consistent with that expected for HO.

For the DMSO experiment;

$$Y_{DMSO} = \frac{Y_0 (k_{DMSO}[DMSO])}{k_{DMSO}[DMSO] + k_{3ap}[3ap]} \qquad \text{II.7}$$

Which under DMSO concentrations ([DMSO] ≥ 1.5 mM), much greater that the concentration of 3ap, ([3ap] = 50 μM), (Figure A1 and A2, Appendix A), and under quantitative scavenging of the methyl radical by 3ap (Figure A3, Appendix A) reduces to,

$$Y_{DMSO} = Y_0 \qquad \text{II.8}$$

For the experiment with methane;

$$Y_{CH4} = \frac{Y_0 (k_{CH4}[CH_4])}{k_{CH4}[CH_4] + k_{3ap}[3ap]} \qquad \text{II.9}$$

Comparing the product yields;

$$\frac{Y_{DMSO}}{Y_{CH4}} = 1 + \frac{k_{3ap}[3ap]}{k_{CH4}[CH_4]} \qquad \text{II.10}$$

Similarly, on rearrangement,

$$R_{\frac{3ap}{CH4}} = \frac{k_{3ap}}{k_{CH4}} = \left[\frac{Y_{DMSO} - Y_{CH4}}{Y_{CH4}}\right] \frac{[CH4]}{[3ap]} \qquad \text{II.11}$$

Comparison of II yields in the DMSO and methane experiments carried out under identical conditions (Figure 2.8), but with a saturating concentration of DMSO, gave an experimental value of $Y_{DMSO}/Y_{CH4} = 3.4 \pm 0.4$ very close to the expected literature value of $Y_{DMSO}/Y_{CH4} = 3.0$, obtained using known values of the rate constants of the reaction of HO with 3ap and CH_4 (Table B2, Appendix B).

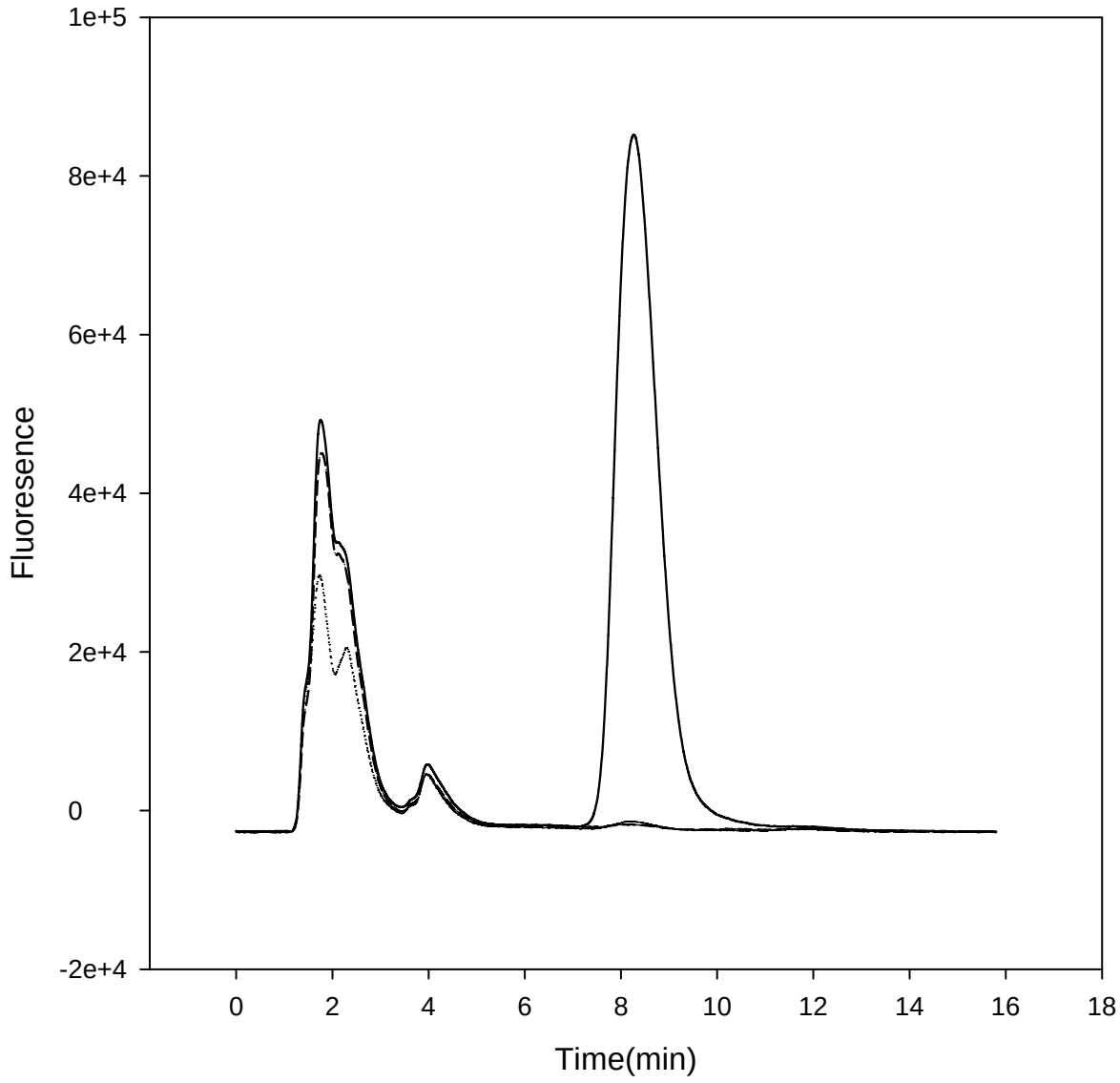


Figure 2.7. Chromatogram showing II formation in the Fenton reaction in the presence of methane and 3ap under anaerobic conditions. Reaction conditions; [3ap] = 50 μM; [CH_4] = 1.5 mM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) and allowed to continue for 15 minutes. In standard reaction (——); standard reaction without Fe (II) (– – –); and standard reaction without DMSO (.....).

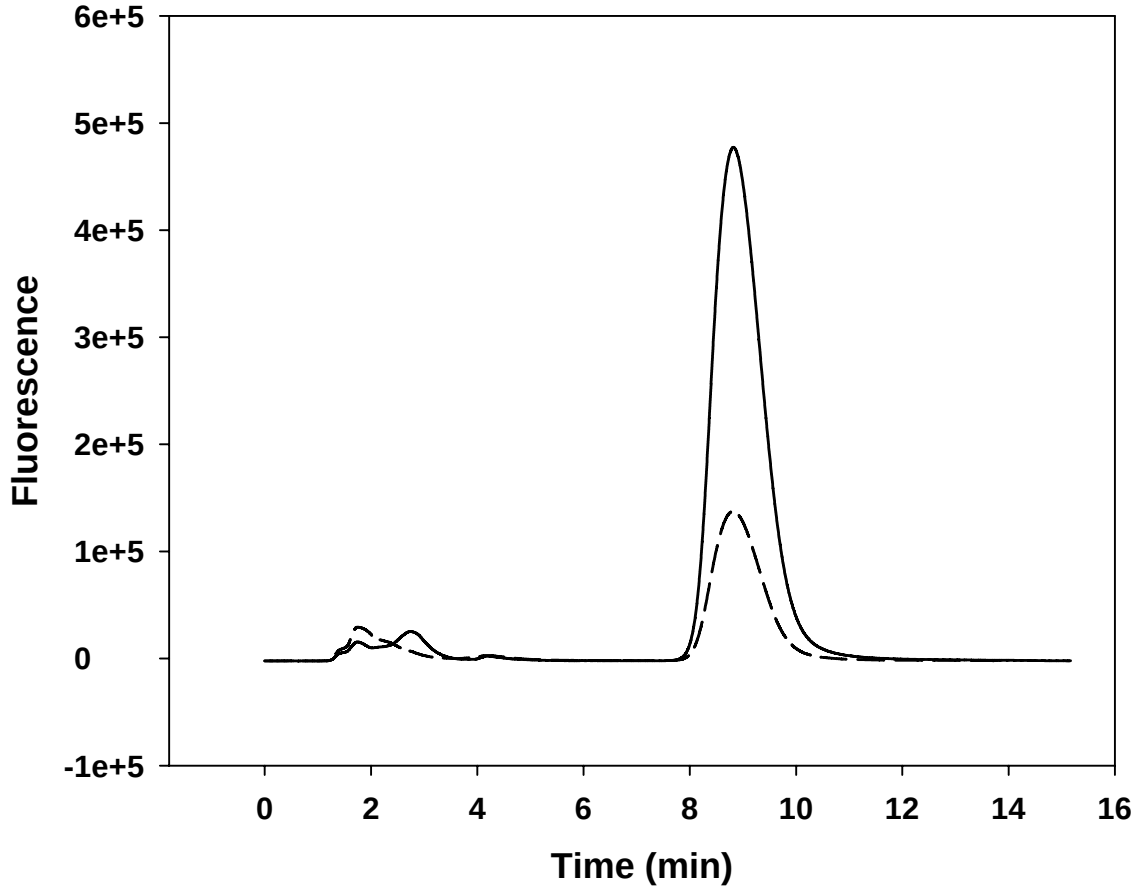


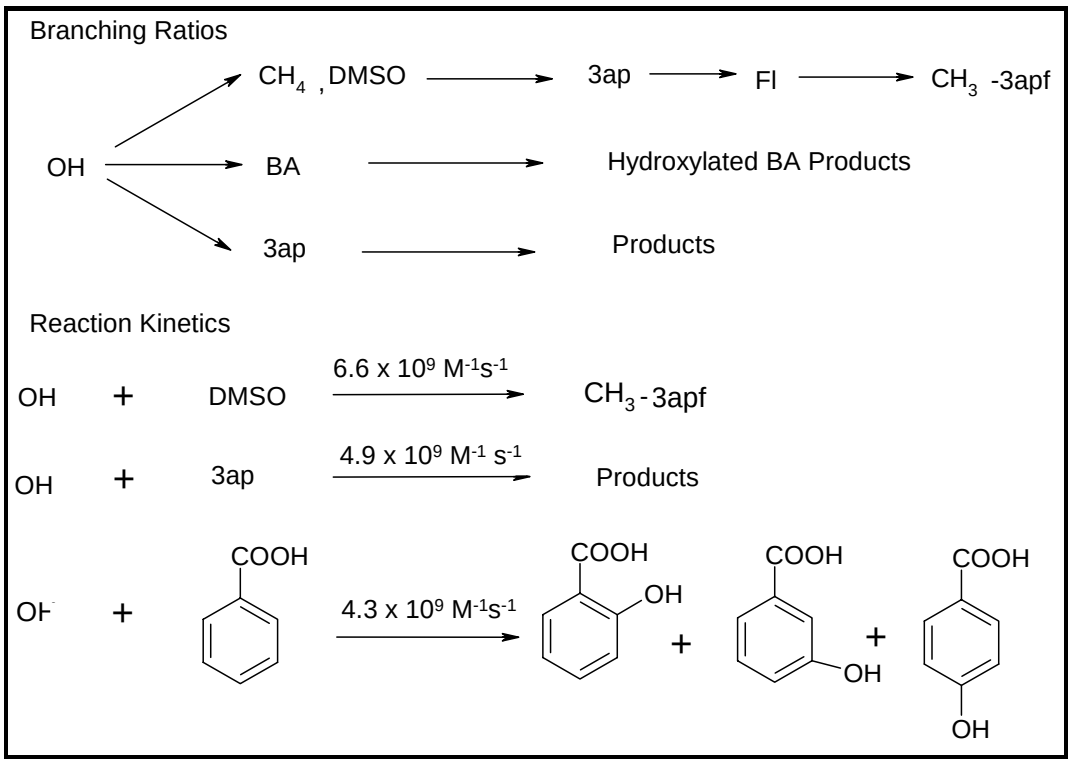
Figure 2.8. Chromatograms showing relative yields of II for the Fenton reaction in presence of either CH_4 or DMSO both at (1.5 mM) under anaerobic conditions. Standard reaction conditions; [3ap] = 50 μM, [H_2O_2] = 150 μM, [Fe (II)] = 10 μM, in 100 mM phosphate buffer at pH 4.2. In this reaction, (——) standard reaction with DMSO, (- - -) standard reaction with methane. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents. The reaction was then allowed to continue to completion (for 15 minutes) before it was derivatized and injected onto the HPLC.

2.3.1.4 Benzoic Acid Experiments

As a further test for the involvement of HO in Fenton reaction, benzoic acid was used as an additional competitor for the intermediates. The advantages of using benzoic acid are two fold; first, it reacts rapidly with HO (Appendix A2), making it easier to compete with the primary scavenger(s). Secondly, in its reaction with HO, BA forms distinct hydroxylated benzoic acid products and their presence in solution also serves as an indicator of HO involvement.

Experiments with DMSO

The yield of II under experimental conditions where sufficiently high concentrations of benzoic acid are used so as to compete effectively with the primary scavenger(s) DMSO or methane can be illustrated by the branching ratio (Scheme 2.2) which also shows the rate constants of the reaction of HO with BA, 3ap and DMSO. The scheme also shows the hydroxylated benzoic acid products expected in a reaction of BA with HO and whose presence in the solution would also be an indication of HO involvement.



Scheme 2.2. Expected branching ratios for the competition reaction involving benzoic acid at fixed concentrations of DMSO and nitroxide. Rate constant values of the reaction of HO with DMSO and BA obtained from literature (Table B2, Appendix B), whereas that with 3ap obtained experimentally using nitrate photolysis (this study).

II formation via the DMSO route in presence of BA is given by,

$$\mathbf{Y_{BA}} = \frac{\mathbf{Y_0\ (k_{DMSO}[DMSO])}}{\mathbf{k_{DMSO}[DMSO] + k_{3ap}[3ap] + k_{BA}[BA]}} \qquad \text{II.12}$$

whereas, product formation in the absence of BA is given by;

$$\mathbf{Y_{DMSO}} = \frac{\mathbf{Y_o\ \ (k_{DMSO}[DMSO])}}{\mathbf{k_{DMSO}[DMSO] + k_{3ap}[3ap]}} \qquad \text{II.13}$$

The ratio of the yield of II in the presence of BA and absence of BA, will be given by

$$\frac{Y_{BA}}{Y_{DMSO}} = \frac{k_{DMSO}[DMSO] + k_{3ap}[3ap]}{k_{DMSO}[DMSO] + k_{3ap}[3ap] + k_{BA}[BA]} \qquad \text{II.14}$$

which simplifies to,

$$Y'_{DMSO} = \frac{1}{1 + \left\{ \frac{k_{BA}[BA]}{k_{DMSO}[DMSO] + k_{3ap}[3ap]} \right\}} \qquad \text{II.15}$$

where,

$$Y'_{DMSO} = \frac{Y_{BA}}{Y_{DMSO}} \qquad \text{II.16}$$

For fixed DMSO and 3ap, expression (II.15), is in the form,

$$Y = \frac{1}{1 + p[BA]} \qquad \text{II.17}$$

Therefore, a functional plot of Y'_{DMSO} with increasing concentration of BA at constant [DMSO] and [3ap], gives a fit with

$$p = \frac{k_{BA}}{k_{DMSO}[DMSO] + k_{3ap}[3ap]} \qquad \text{II.18}$$

and

$$k_{BA} = p(k_{DMSO}[DMSO]_{Exp} + k_{3ap}[3ap]_{Exp}) \qquad \text{II.19}$$

where $[DMSO]_{Exp}$ and $[3ap]_{Exp}$ are the experimental concentrations of DMSO and 3ap respectively.

When sufficiently high concentrations of benzoic acid were used so as to compete effectively with both the primary scavenger, DMSO, as well as the nitroxide, II yields decreased with increasing BA (Figure 2.9 and Figure A4, Appendix). This reduction in yield of II is further illustrated in Figure 2.10, in which the experimental data are fit to the non-linear regression represented by function, $f_n = 1/(1+ bx)$.

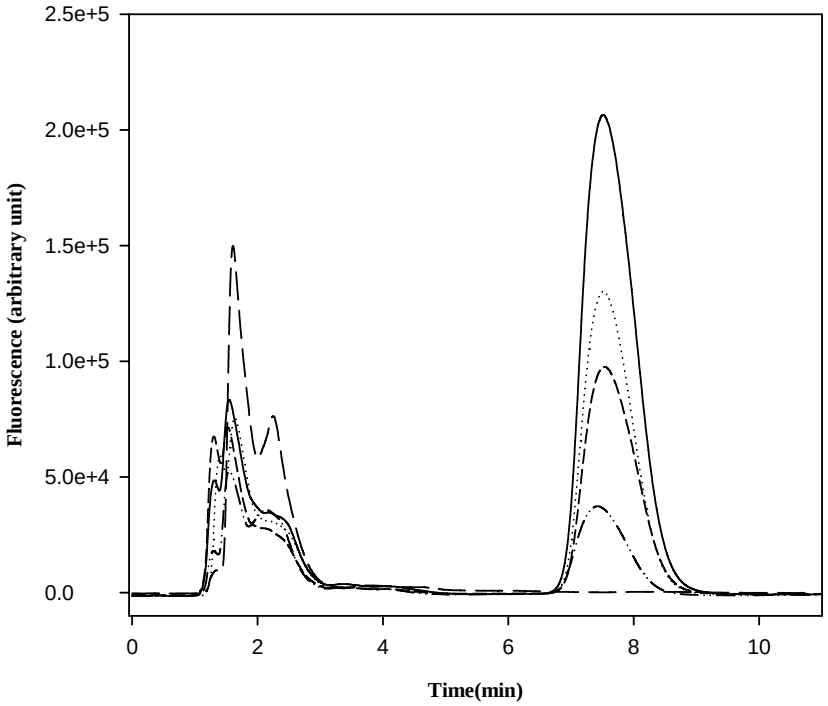


Figure 2.9 Chromatograms showing the decrease in II yield with increasing [BA] at fixed concentrations of DMSO and 3ap. Reaction conditions; [3ap] = 30 μM; [DMSO] = 30 μM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 4.2. The reactions were performed anaerobically at varying conditions whereby; (——) standard reaction with 0.1mM BA, (……) standard reaction with 0. 25 mM BA, (– – –) standard reaction with 0.50 mM BA and, (— · · —) standard reaction with 1.0 mM BA and (—— ——) standard reaction without Fe (II).

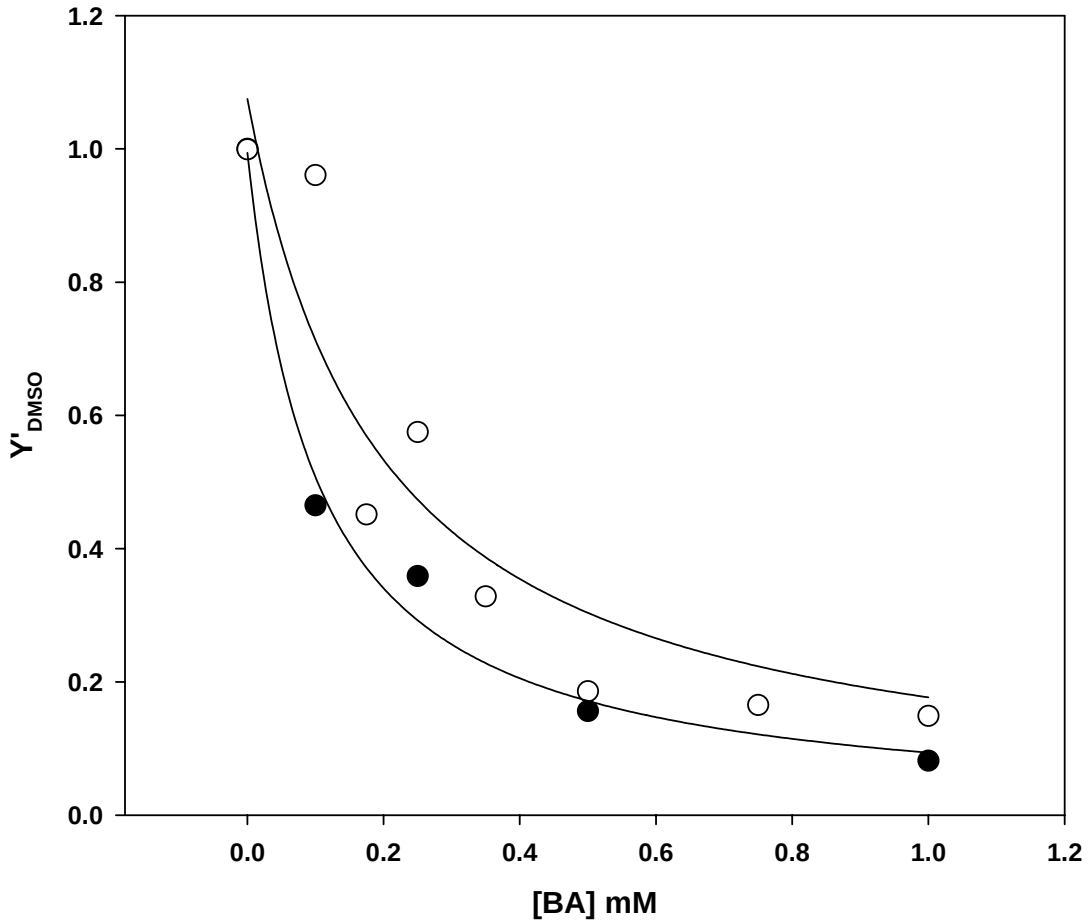


Figure 2.10. The dependence of the ratio of II yield (in the presence and absence of BA, see text) in the presence of fixed concentrations of DMSO and nitroxide. Reaction conditions; 50 μM 3ap; 10 μM Fe (II), 150 μM H_2O_2, in 100 mM phosphate buffer at pH 4.2. The reactions were repeated for; (●) 30 μM DMSO and (○) 50 μM DMSO, they were initiated by addition of the ferrous ions and allowed to continue anaerobically for 15 minutes. The curve represents a non-linear regression of the form $f_n = 1/ (1 + bx)$.

The experimental values of $k_{BA} = (4.3 \pm 0.65) \times 10^9$ $M^{-1}s^{-1}$ and $(2.95 \pm 0.95) \times 10^9$ $M^{-1}s^{-1}$ obtained using expression II.19 and employing a fit of the experimental data to expression II.15, (Figure 2.10, and Table A1, Appendix A), and the known rate constants

of the reaction of HO with DMSO (4.3×10^9 $M^{-1}s^{-1}$, from literature Table B2, Appendix B) and with 3ap (4.9×10^9 M-1s-1, measured experimentally using nitrate photolysis in this study), compares well to the literature value of k_{BA} in the range of $(1.8 - 6.2) \times 10^9$ $M^{-1}s^{-1}$, Table B2, Appendix B).

Experiments with Methane

Similar competition experiments with BA were done with methane in place of DMSO. As with DMSO the yield of II was found to decrease with increasing BA concentration (Figure 2.11).

A similar expression as (II.3) for methane can be derived,

$$\mathbf{Y'_{CH4}} = \frac{\mathbf{1}}{\mathbf{1} + \left\{ \frac{\mathbf{k_{BA}[BA]}}{\mathbf{k_{CH4}[CH4] + k_{3ap}[3ap]}} \right\}} \qquad \text{II.20}$$

which is equivalent to;

$$\mathbf{Y'_{CH4}} = \frac{\mathbf{1}}{\mathbf{1} + \mathbf{q[BA]}} \qquad \text{II.21}$$

whereby,

$$\mathbf{q} = \frac{\mathbf{k_{BA}}}{\mathbf{k_{CH4}[CH_4] + k_{3ap}[3ap]}} \qquad \text{II.22}$$

$$\mathbf{k_{BA}} = \mathbf{q(k_{CH4}[CH4]_{Exp} + k_{3ap}[3ap]_{Exp})} \qquad \text{II.23}$$

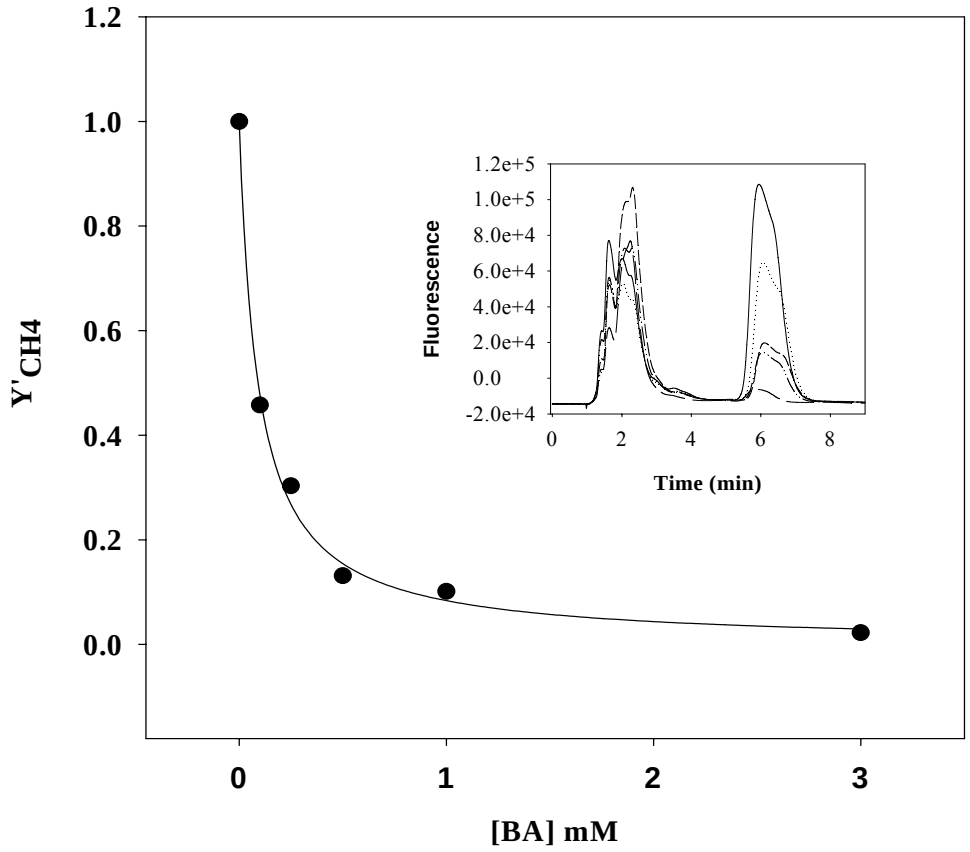


Figure 2.11. The dependency of II yield on BA in the presence of a fixed concentration of methane. Reaction conditions; 1.5 mM CH_4, 10 μM Fe (II), 150 μM H_2O_2, in 100 mM phosphate buffer at pH 4.2. The reaction was initiated by addition of the ferrous ions and allowed to continue anaerobically for 15 minutes. The curve represents a least squares fit to the form $f_n = a/ (1 + bx)$ where a = 1.

Inset: chromatograms showing the decrease in II yield with increasing [BA] at fixed concentrations of CH_4 and 3ap. The reactions were performed anaerobically at varying conditions whereby; (——) standard reaction with 0.1mM BA, (……) standard reaction with 0. 25 mM BA, (– – –) standard reaction with 0.50 mM BA and, (— · · —) standard reaction with 1.0 mM BA and (—— ——) standard reaction with 2.0 mM BA.

With the methane experiment, the experimental value of $k_{BA} = (4.7 \pm 0.3) \times 10^9$, compares closely with the literature value in the range of (1.8 to 6.2) x 10^9, (Table B2, Appendix B) and reinforces the fact that HO is the oxidizing intermediate involved in this reaction.

Product Identification

As a further test using of the intermediates involved in the Fenton reaction, the Fenton reaction was performed in presence of BA and 3ap. In this experiment, a standard solution of 2-hydroxylated benzoic acid (2-OH-BA) was co-eluted with products formed in the benzoic acid experiment so as to determine whether any 2-OHBA products were formed in the reaction of BA with free OH, which would co-elute with the standard 2-OHBA. At the appropriate HPLC conditions, an increase in the product formed in the experiment when a spike of the 2-OHBA was added was observed (Figure 2.12).

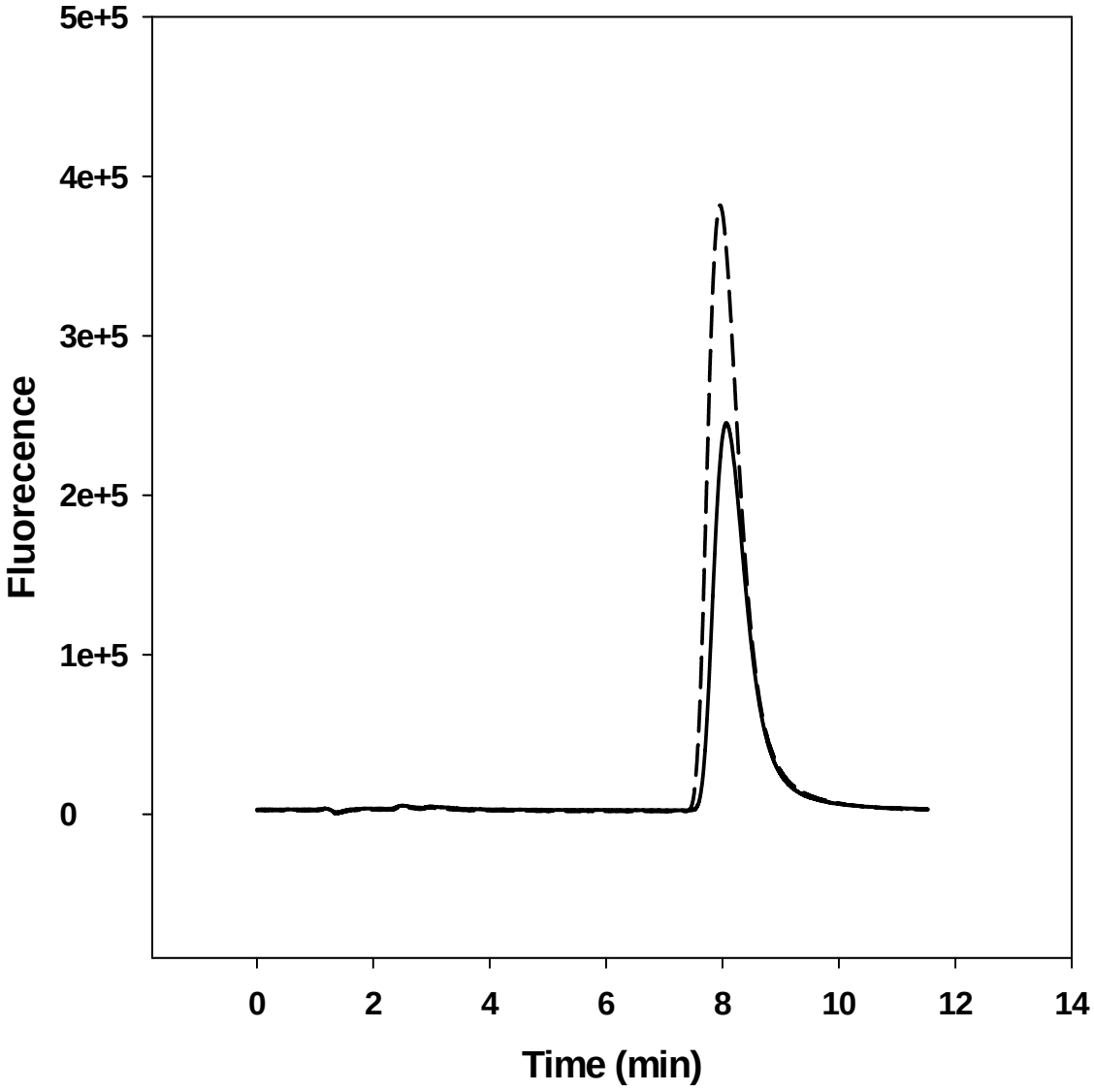


Figure 2.12 Chromatograms showing 2-hydroxylated benzoic acid (2-OH BA) product formation in a reaction containing BA. Other reaction conditions; [3ap] = 50 μM; [DMSO] = 30 μM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 4.2. The reactions were done anaerobically. Here the solid line (——) portrays the product from the reaction and the dashed line (— —) portrays this product spiked with 1.9 μM of 2-OHBA. From this standard addition, the yield of 2-OHBA in the non-spiked sample was 3.8 μM.

2.3.1.5 Metal ion concentration dependence of the yield of II

The purpose of this study was to evaluate the yields of II with increasing metal ion concentrations so as to give an indication of the reaction stoichiometry. It is also expected that from such experimentation, one may be able to determine the extent of HO involvement under the given experimental conditions. When the concentration of Fe (II) was varied between 5.0 μM and 25 μM, an increase in II yield was observed (Figure 2.13). The experiments were performed in both 2 mM and 100 mM phosphate buffer Figure (2.13 and Figures A5 and A6, Appendix), and from these results, it is evident that II yield under these conditions is 90%.

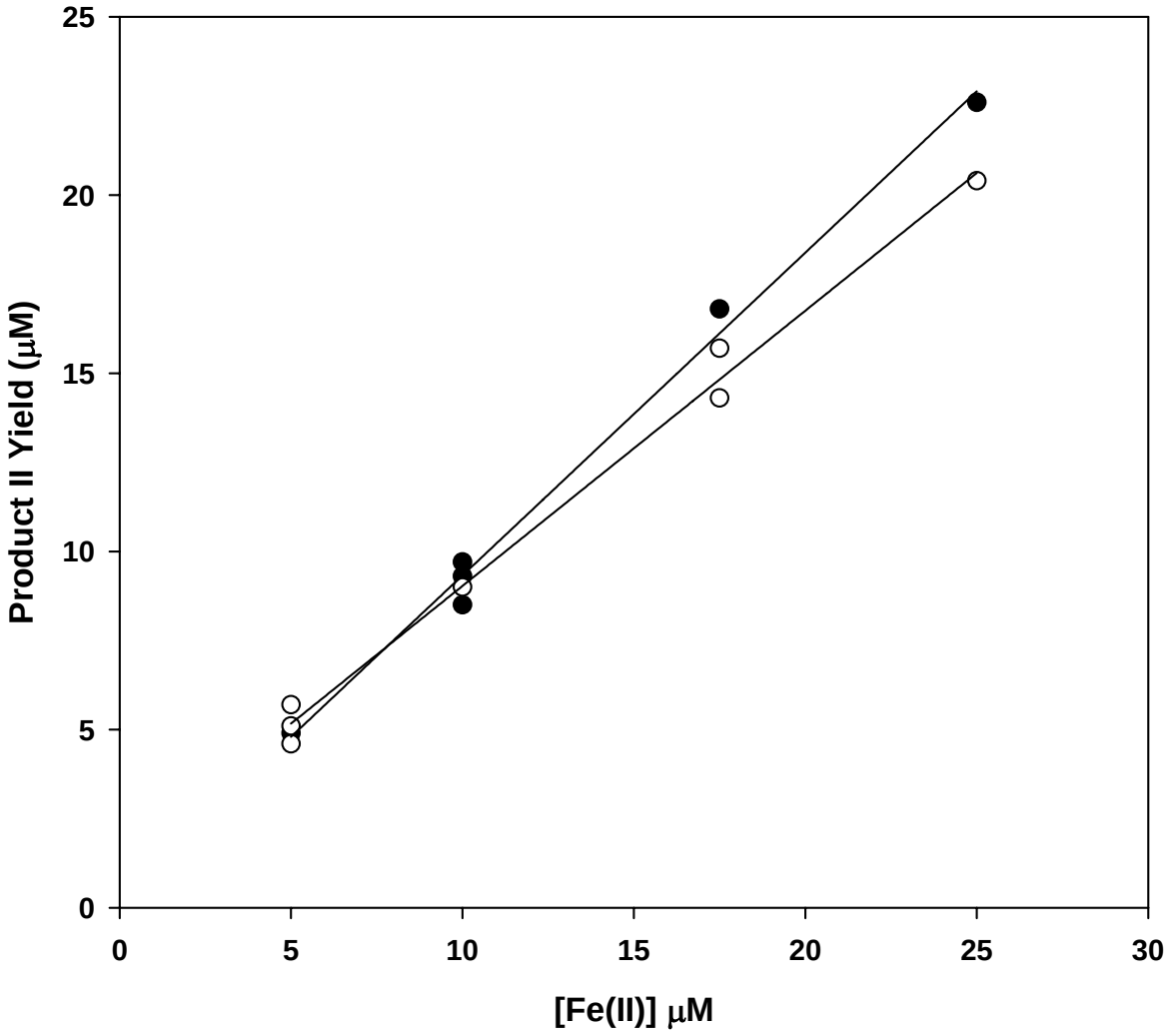


Figure 2.13 Product formation with increasing concentration of Fe (II). Reaction conditions; [3ap] = 50 μM; [DMSO] = 10 mM; [H_2O_2] = 150 μM, in (●) 2 mM phosphate buffer and (○)100 mM phosphate buffer at pH 4.2. Reaction was initiated by addition of the metal ions of varying concentration for both experiments. The percentage product yield in this case was 90 ± 4 (based on the slope of the above figure), for the reaction in 2 mM phosphate buffer.

2.3.2 Experiments at Neutral pH

Most reactions in biological systems occur at physiological pH which is close to neutral pH. Similarly, a good number of environmental reactions also take place at close to neutral pH. For these reasons, it was important carry out a study of the Fenton reaction at neutral pH and thereby determine the nature of the species involved under these conditions, which would in essence have more relevance to the two systems.

2.3.2.1 Fenton Experiments with DMSO and methane

Reactions carried out anaerobically in 100 mM phosphate buffer at pH 7.4 yielded a large signal (II) (Figure 2.14), identical to that obtained at lower pH for both DMSO (not shown) and methane. This gave an indication that even at neutral pH a sufficiently strong oxidizing species, capable of oxidizing DMSO and methane to yield methyl radicals and similar to that observed at low pH was involved.

A comparison of II yield in the DMSO and methane experiments carried out under otherwise identical conditions at neutral pH 7.4 gave a Y_{DMSO}/Y_{CH4}, (equation II.2) value of 3.2 ± 0.7 which compared well with the literature value of 3.0 (Figure 2.15) as well as the value obtained at lower pH (3.4 ± 0.4) and was consistent with that expected for HO.

2.3.2.2 Competition Experiments

Competition studies with the nitroxide and benzoic acid at neutral pH were also performed, and the yield of II was found to decrease with increasing concentration of the nitroxide (Figures 2.16 and Figures A7 and A8, Appendix) or benzoic acid, (Figures 2.17

and 2.18) respectively. For the nitroxide competition studies, the average experimental value, of $k_{3ap}/k_{BA} = 0.92 \pm 0.29$ (obtained from an average of experimental values, Table 2.2) compared well with the value of 0.74 ± 0.09 obtained in the case of the nitrate photolysis experiment. In the case of the BA, experiments performed in presence of either DMSO or methane, (Figures 2.17 and 2.18), gave experimental values of $k_{BA} = 4.7 \pm 0.36$ and $k_{BA} = 6.6 \pm 0.4$ respectively, that were comparable to the literature value in the range of $k_{BA} = (1.8 – 6.2) \times 10^9$ for the reaction of HO with BA, (Table 2.3), and were a further indication of HO involvement in Fenton reactions at neutral pH.

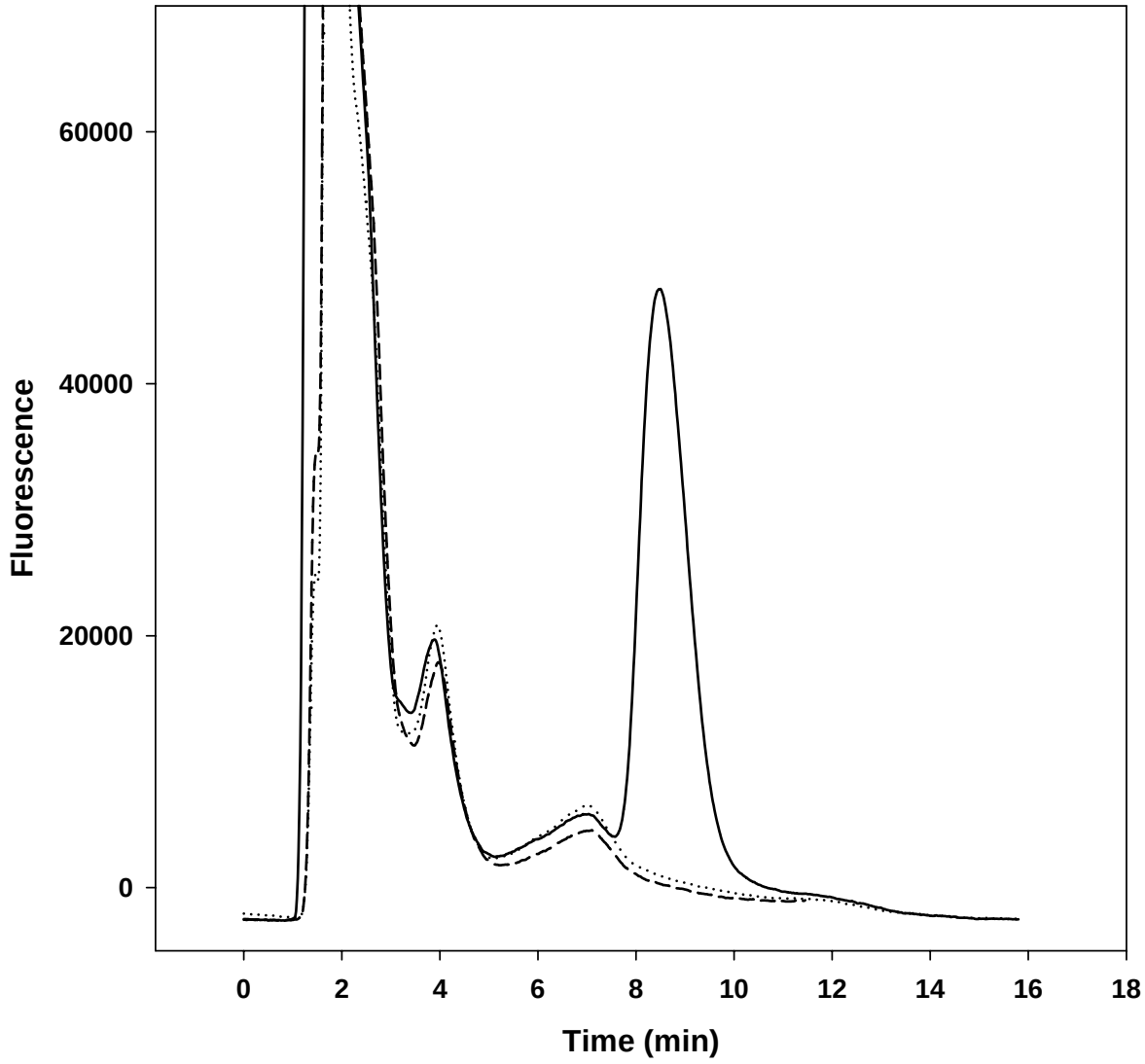


Figure 2.14. Chromatogram showing II formation in the Fenton reaction in the presence of methane and 3ap under anaerobic conditions. Reaction conditions; [3ap] = 50 μM; $[CH_4]$ = 1.5 mM; $[H_2O_2]$ = 150 μM, in 100 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 μM Fe (II) and allowed to continue for 15 minutes. In standard reaction (——); standard reaction without Fe (II) (– – –); and standard reaction without DMSO (.....).

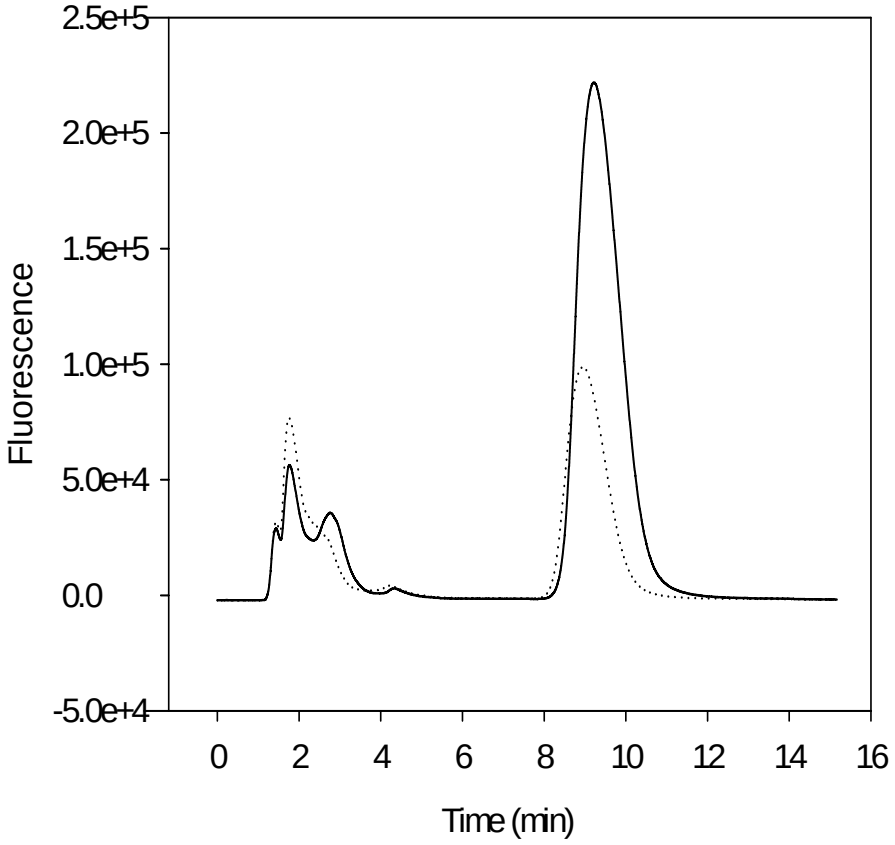


Figure 2.15. Chromatograms showing relative yields of II for the Fenton reaction in presence of either CH_4 or DMSO both at (1.5 mM) under anaerobic conditions. Standard reaction conditions; [3ap] = 50 μM, [H_2O_2] = 150 μM, [Fe (II)] = 10 μM, in 100 mM phosphate buffer at pH 7.4. In this reaction, (——) standard reaction with DMSO, (- - -) standard reaction with methane. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents. The reaction was then allowed to continue to completion (for 15 minutes) before it was derivatized and injected onto the HPLC.

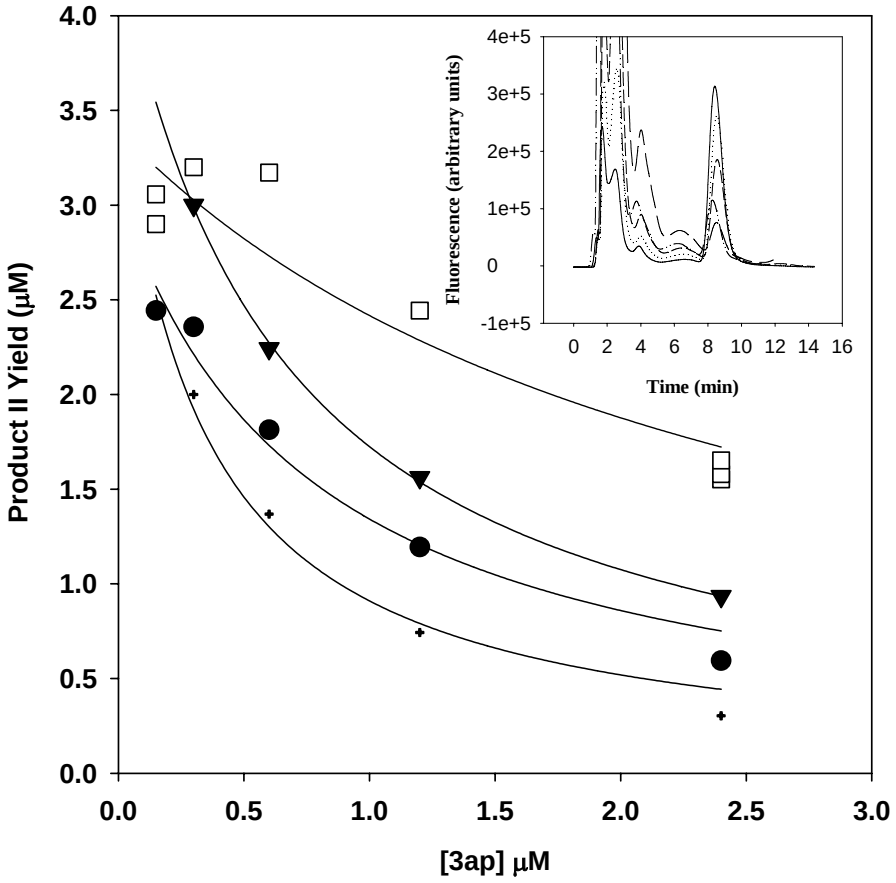


Figure 2.16. The dependence of II yield on [3ap] in presence of a fixed concentration of DMSO. Reaction conditions; 150 μM H_2O_2, 10 μM Fe (II), in 100 mM phosphate buffer at pH 7.4. The reaction was initiated by addition of the ferrous ions and allowed to continue anaerobically for 15 minutes. The curves represent a non-linear regression of the form $f_n = a/ 1 + bx$. The reaction was performed at various concentrations of DMSO; (+) 0.25 mM, (●) 0. 05 mM, (▼) 1.0 mM and (□) 2.0 mM.

Inset, chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO (500 μM). The experiments were performed with varying amounts of 3ap; (——) 0.15 mM, (.....) 30 mM, (– – –) 0.60, an15 d (– · · –) 1.2 mM and (—— ——) 2.4 mM respectively. For the 2mM experiment, at the lower 3ap concentrations, the high concentration of DMSO used render it difficult for the 3ap to cause (initially) any significant reduction in II yield.

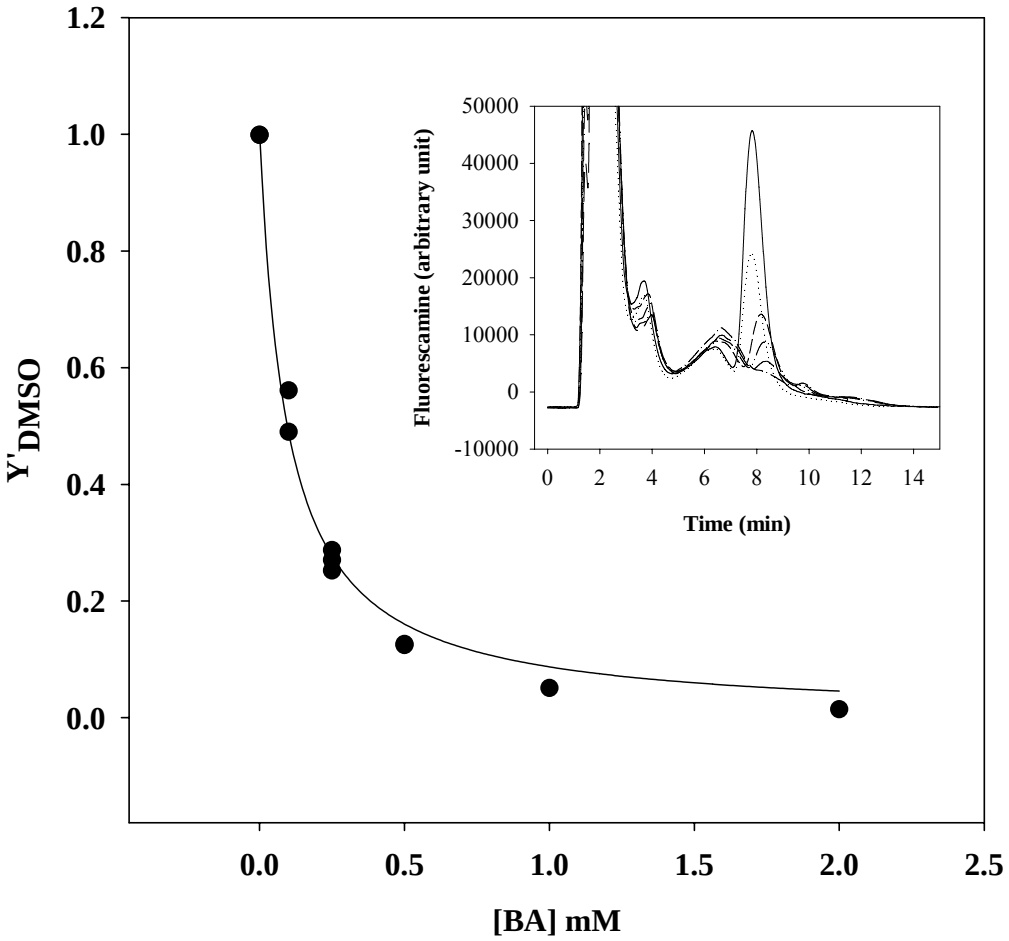


Figure 2.17. The dependence of II yield on BA in presence of a fixed concentration of DMSO and 3ap. Reaction conditions; 50 µM DMSO, 50 µM 3ap, 10 µM Fe (II), 150 µM H_2O_2, in 100 mM phosphate buffer at pH 7.4. The reaction was initiated by addition of the ferrous ions and allowed to continue anaerobically for 15 minutes. The curve represents a non-linear regression of the form $f_n = a/ 1 + bx$, whereby a = 1.
Inset, chromatograms showing the decrease in II yield with increasing [BA] at fixed concentrations of DMSO and 3ap. The reactions were performed anaerobically at varying conditions whereby; (——) standard reaction without BA, (……) standard reaction with 0.1mM BA, (– – –) standard reaction with 0. 25 mM BA, (— · · —) standard reaction with 0.50 mM BA and, (—— ——) standard reaction with 1.0 mM BA and (—·—·—) standard reaction with 2.0 mM BA.

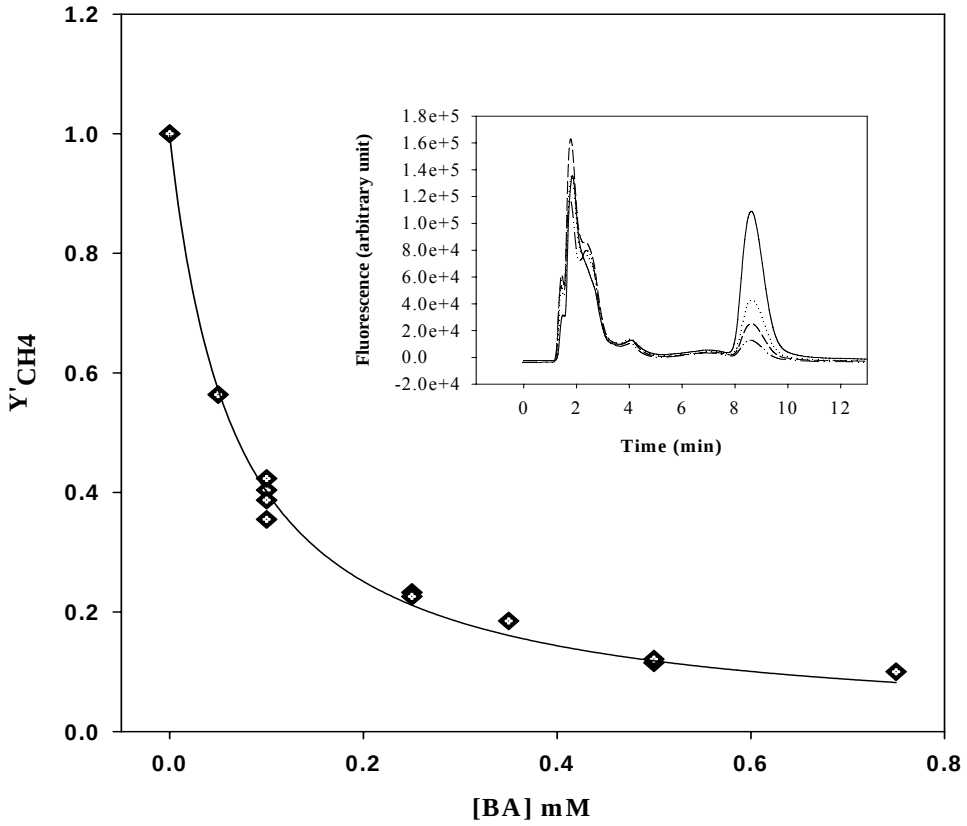


Figure 2.18. The dependence of II yield on BA in presence of a fixed concentration of methane and 3ap. Reaction conditions; 1.5 mM CH_4, 50 µM 3ap, 10 µM Fe (II), 150 µM H_2O_2, in 100 mM phosphate buffer at pH 7.4. The reaction was initiated by addition of the ferrous ions and allowed to continue anaerobically for 15 minutes. The curve represents a non-linear regression of the form $f_n = a/ 1 + bx$ whereby a = 1.
Inset, chromatograms showing the decrease in II yield with increasing [BA] at fixed concentrations of CH_4 and 3ap. The reactions were performed anaerobically at varying conditions whereby; (——) standard reaction without BA, (……) standard reaction with 0.1mM BA, (– – –) standard reaction with 0. 25 mM BA, and (— · · —) standard reaction with 0.50 mM BA.

Table 2.2 Summary of findings for the Fenton and nitrate photolysis studies with the nitroxide

Experimental Study	Product Comparisons	Competition Experiments	
	Y_{DMSO}/Y_{CH4}	[DMSO] mM	$R_{3ap/DMSO} = k_{3ap}/k_{DMSO}$
Nitrate			0.74 ± 0.09
Fenton, pH 4.2	3.4 ± 0.40	0.50	1.2 ± 0.5
Fenton, pH 7.4	3.2 ± 0.70	0.25 0.50 1.0 2.0	0.75 ± 0.15 0.64 ± 0.15 1.52 ± 0.08 0.80 ± 0.18
Literature	3.0		0.74

Table 2.3 Summary of findings for the Fenton Reaction Studies with BA

Reactants	**Conditions**		**$k_{BA} = p\{(k_{DMSO}[DMSO]+k_{3ap}[3ap]\}$ (x 10^9) / $M^{-1}s^{-1}$**	
	pH	**[DMSO] or [CH_4]**	**Experimental**	**Literature****
DMSO	pH 4.2	0.03 mM	4.3 ± 0.65	1.8
		0.05 mM	2.95 ± 0.95	
	pH 7.4	0.03 mM	4.7 ± 0.36	4.3 (≤ 3)
CH_4	pH 4.2	1.5 mM	4.7 ± 0.3	5.7 (≈ 7)
	pH 7.4	1.5 mM	6.6 ± 0.4	6.2

Values obtained from Neta P.; Huie R. E; Buxton G. V.; Helman W. P.; Millard W. G and Ross A. B. *NIST standard reference Database,* Ver. 3.0, Gaithersburg, MD **1998. Values in brackets represent pH conditions. * p fitting parameters obtained from least squares fit of experimental values to expression II.3, (Figures 2.10, 2.11, 2.17 and 2.18) and are contained in table A2 (Appendix).

2.3.2.3 Metal ion dependence studies

Studies performed at varying metal ion concentration gave II yields that were much lower than those obtained at lower pH (Figure 2.19 and Figure A9, Appendix). Under these conditions, II yield was only 20 % compared to the greater than 90 % obtained at lower pH. The reduction in II yield was attributed to the fact that since the experiments were done in phosphate buffer, which may not bind the metal ions strongly enough to prevent oxidation by oxygen, the reduction in II yield may have been due to a partial oxidation of Fe (II) to Fe (III), which would then easily hydrolyze to the insoluble form thereby reducing the amount of available metal in the reaction.

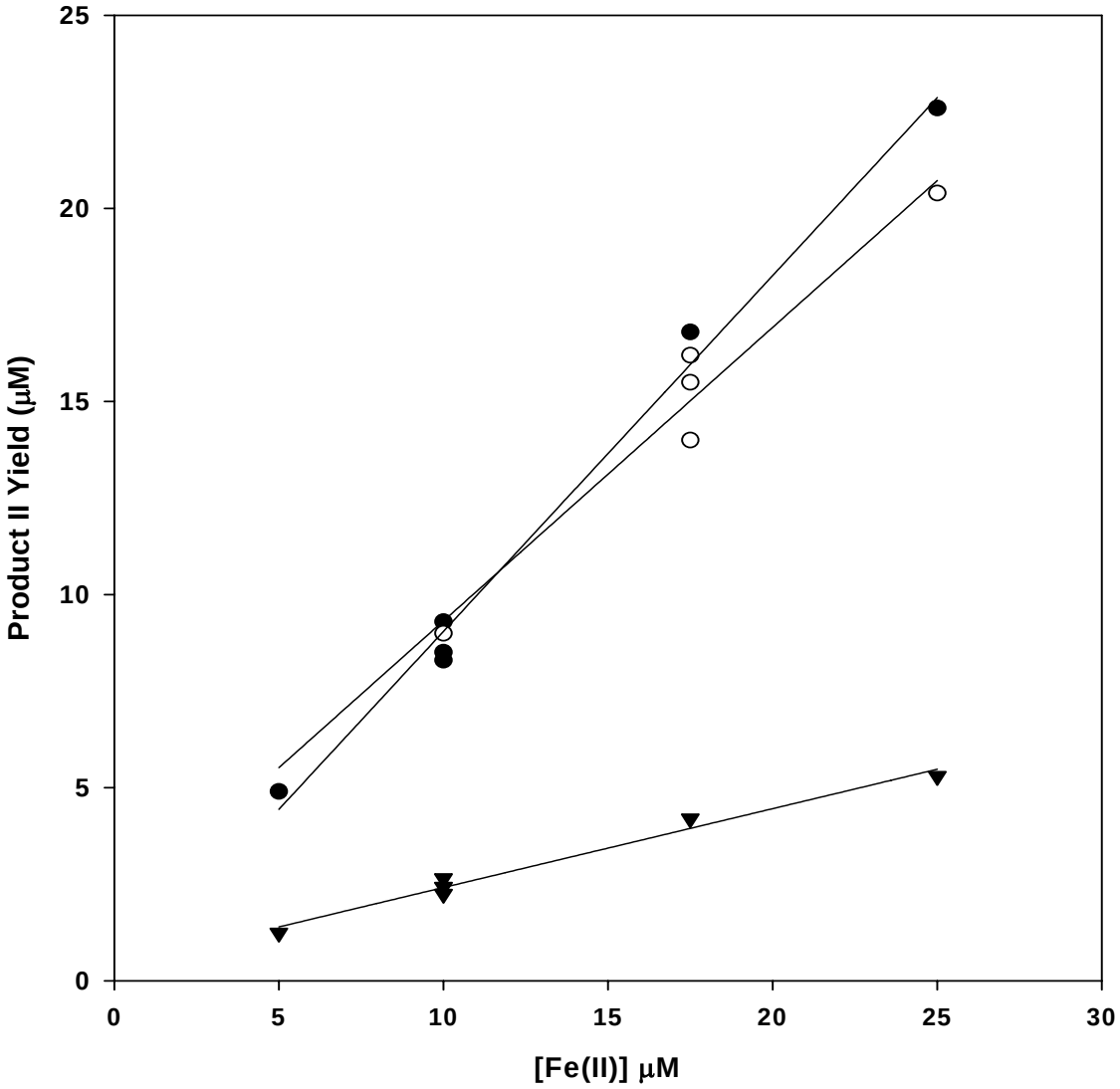


Figure 2.19. II formation with increasing concentration of metal ion. Reaction conditions; [3ap] = 50 μM, [DMSO] = 10 mM, [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 7.4 (▼). Reaction was initiated by addition of the metal ions of varying concentration. The percentage II yield in this case was 20.4 ± 1.4. Experiments carried out with 2 mM (●) and 100 mM (○) phosphate buffer at pH 4.2 are included for comparison.

2.3.3 Experiments with Fe (III)

Studies have suggested that one of the ways to produce high valent metal species is by oxidation of the ferric ions by hydroxyl radicals (Rush & Kopppenol 1986). In this case, if the Fenton reaction is carried out in presence of high concentrations of Fe (III) one may expect generation of the high valent metal species.

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + HO^- + HO^{\bullet} \qquad 1.1$$

Followed by

$$HO^{\bullet} + Fe(III) \longrightarrow Fe(IV) + OH^- \qquad 1.23$$

Experiments with DMSO

Since Fe (III) is in large excess, it can compete with both DMSO and 3ap for the HO radicals.

$$OH + DMSO \xrightarrow{6.6 \times 10^9\ M^{-1}s^{-1}} {}^{\bullet}CH_3 \longrightarrow \longrightarrow \longrightarrow CH_3\text{-}3apf$$

$$OH + 3ap \xrightarrow{4.89 \times 10^9\ M^{-1}s^{-1}} Products$$

$$OH + Fe(III) \xrightarrow{8.5 \times 10^7\ M^{-1}s^{-1}} Fe(IV) \xrightarrow{?} DMSO \longrightarrow \longrightarrow \longrightarrow CH_3\text{-}3apf$$

When the Fenton reaction was carried out in presence of DMSO, 3ap and 10 mM Fe (III) a significant yield of II was observed (Figure 2.20). The yield of II was equal to that obtained when the experiment was done in absence of Fe (III). These results raised a

few possibilities; first, either the presence of iron (III) has no effect on the Fenton reaction in presence of DMSO, second, the species formed

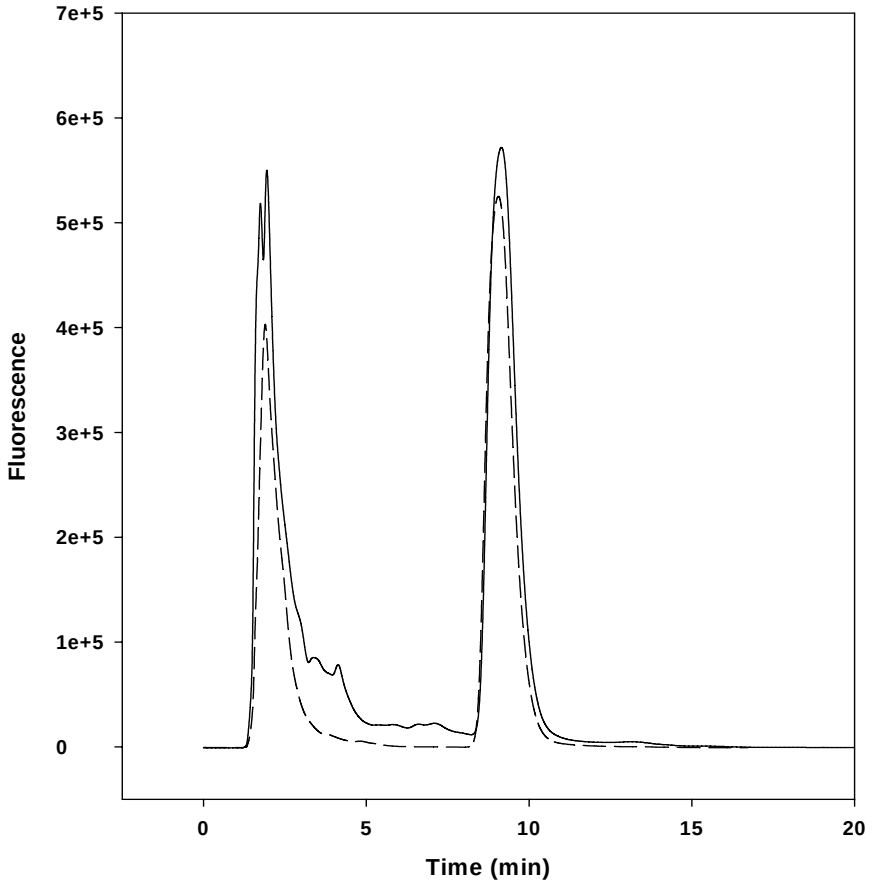


Figure 2.20 chromatograms illustrating II formation under anaerobic conditions in a standard Fenton reaction; with Fe (III) (- - -), and without Fe (III) (——) in presence of DMSO and 3ap. Standard reaction conditions; 50 μM 3ap, 100 μM DMSO, 150 μM H_2O_2, 10 mM Fe (III), in 2 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents and allowed to proceed to completion (15 minutes), before it was derivatized and injected onto the HPLC.

by the reaction of Fe (III) and HO oxidize DMSO to yield methyl radicals resulting in minimal change in the total II yield; thirdly, the reduction in the yield of II is much lower than 50 % and may be masked by poor experimental reproducibility.

In order to address the third issue, the Fenton reactions with and without Fe (III) were repeated three times, with good reproducibility (Figure 2.21). The experimental errors obtained in the standard Fenton reaction and in the Fenton reaction in presence of Fe (III) were 3.0% and 8.0 % respectively. It was also important to test whether the large amounts of Fe (III) would in any way interfere with the sample derivatization process. To examine this possibility, Fe (III) was added to an aliquot from a standard Fenton reaction before derivatization. When this mixture was derivatized, it gave II yields identical to those obtained from an aliquot of the standard Fenton reaction without any Fe (III) addition (Figure 2.22). This result indicated that the presence of Fe (III) had little or no effect on the derivatization process.

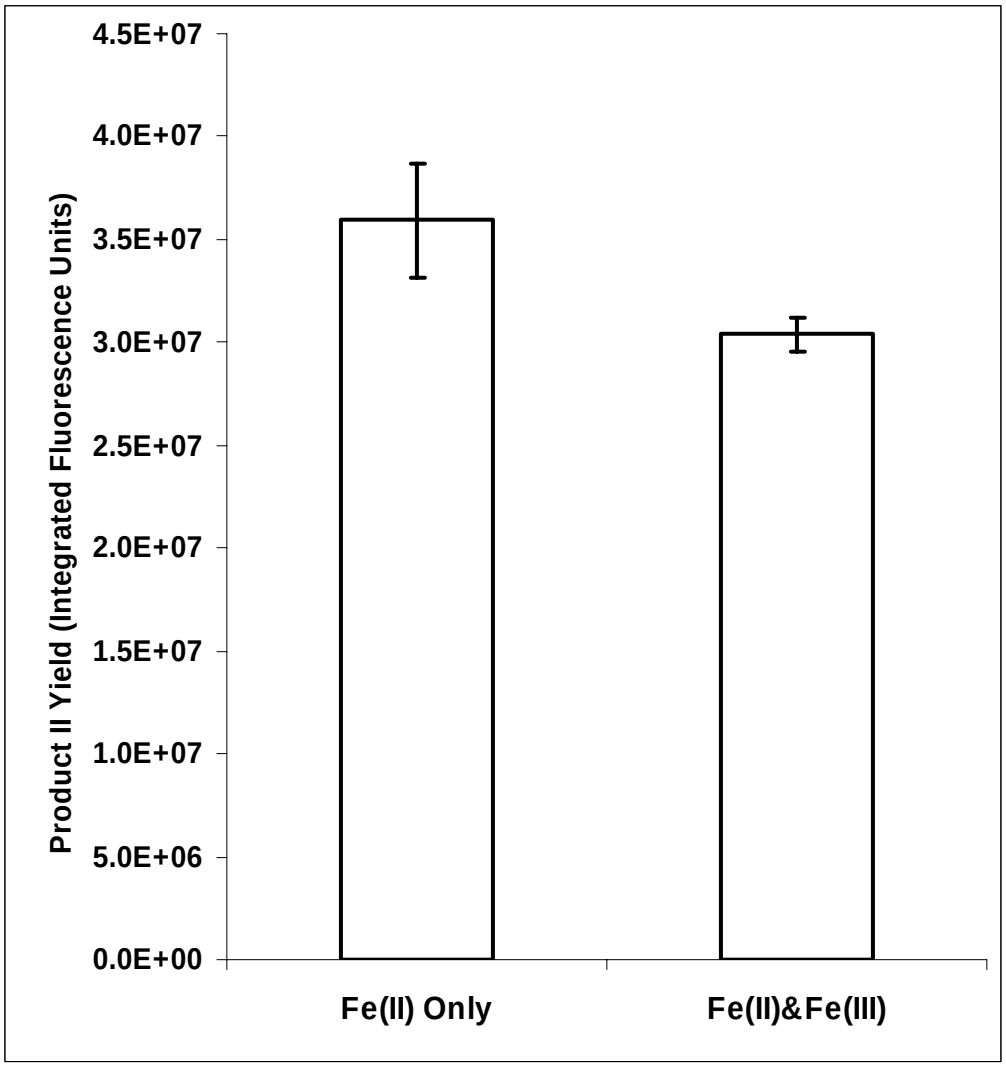


Figure 2.21. II yields in a standard Fenton reaction in presence of DMSO with and without 10 mM Fe (III). Standard reaction conditions; 50 μM 3ap, 100 μM DMSO, 150 μM H_2O_2, 10 μM Fe (II), in 2 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents and allowed to proceed to completion (15 minutes). The Fe (III) was added to the standard Fenton reaction before it was derivatized.

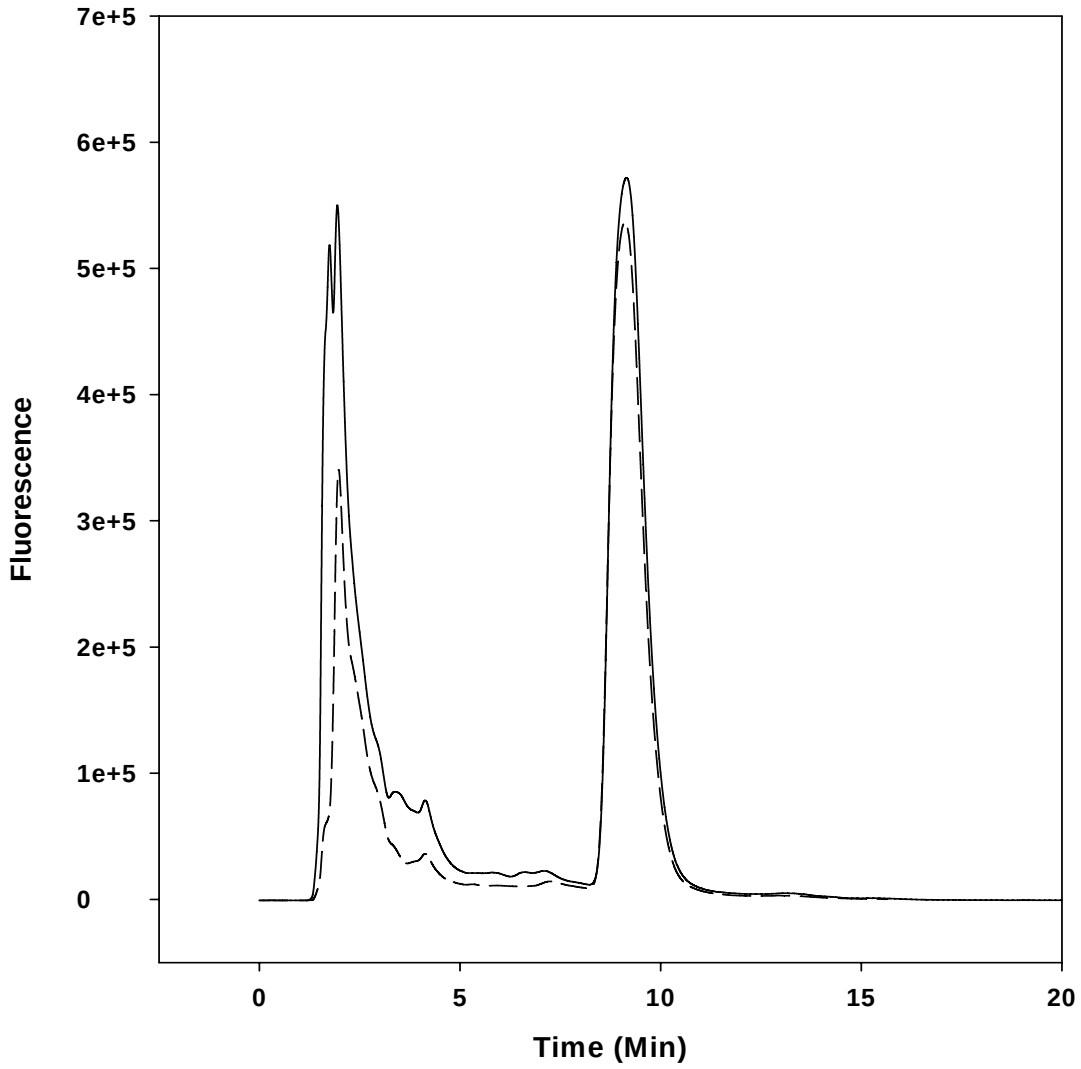


Figure 2.22. Chromatograms indicating II formation in a standard Fenton reaction derivatized in the absence (——) and presence (- - -) of 10 mM Fe (III) and in the presence of DMSO and 3ap. Standard reaction conditions; 50 μM 3ap; 100 μM DMSO; 150 μM H_2O_2, 10 μM Fe (II), in 2 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents and allowed to proceed to completion (15 minutes), before it was derivatized and injected onto the HPLC.

Experiments with methane

In order to test whether the intermediate formed by the reaction of Fe (III) with HO reacted or oxidized DMSO to yield methyl radicals, methane was used in the experiment in place of DMSO. Because only sufficiently strong species may oxidize methane, unlike DMSO, comparison of the methane and DMSO experiments carried out under identical conditions may give an indication of the oxidizing ability of these species.

$$OH + CH_4 \xrightarrow{1.2 \times 10^8\ M^{-1}s^{-1}} {}^{\cdot}CH_3 \rightarrow \rightarrow \rightarrow CH_3\text{-}3ap$$

$$OH + 3ap \xrightarrow{4.89 \times 10^9\ M^{-1}s^{-1}} Product$$

$$OH + Fe(III) \xrightarrow{8.5 \times 10^7\ M^{-1}s^{-1}} Fe(IV) \xrightarrow{?} CH_4 \rightarrow \rightarrow \rightarrow CH_3\text{-}3ap$$

When the Fenton reaction was carried out with methane in place of DMSO in the presence of a large excess of Fe (III), the formation of II yield was observed (Figure 2.23), but at yields much lower than those obtained in a standard Fenton reaction in the absence of Fe (III). An expression similar to II.12, can be derived for the standard Fenton reaction in presence of Fe (II),

$$Y_{DMSO} = \frac{Y_0\ (k_{CH4}[CH_4])}{k_{CH4}[CH_4] + k_{3ap}[3ap] + k_{Fe(III)}[Fe(III)]} \qquad \text{II.24}$$

On the other hand, in the absence of Fe (III), expression II.13 may be used. Using these expressions (II.13 and II.24), the known rate constants above (Table B2, Appendix B), for the reaction of HO with 3ap, Fe (III) and CH_4 as well as the concentrations of 3ap (50 μM), CH_4 (1.5 mM) and Fe (III) (10 mM) used (Figure 2.23), the expected yield of II in

presence of Fe (III) would be only 14 % compared to 42 % yield in absence of Fe (III). This represents a 33 % reduction in yield which is close to the experimental value of 43 ± 4.9 % for the reduction in II yield in presence of Fe(II) obtained from a series of independent experiments using similar conditions (Figure 2.23 and Figure 2.24).

These observations indicated that the intermediate species formed in the reaction of Fe (III) with HO oxidizes DMSO to yield methyl radicals but are not capable of oxidizing methane in a similar manner to yield methyl radicals. A comparison between the yields of II in the presence of DMSO and CH_4 thus provide an excellent way of discriminating between high–valent metal species and HO in these reactions and agree with our previous observations regarding the involvement of HO in the Fenton reaction under all conditions of our study.

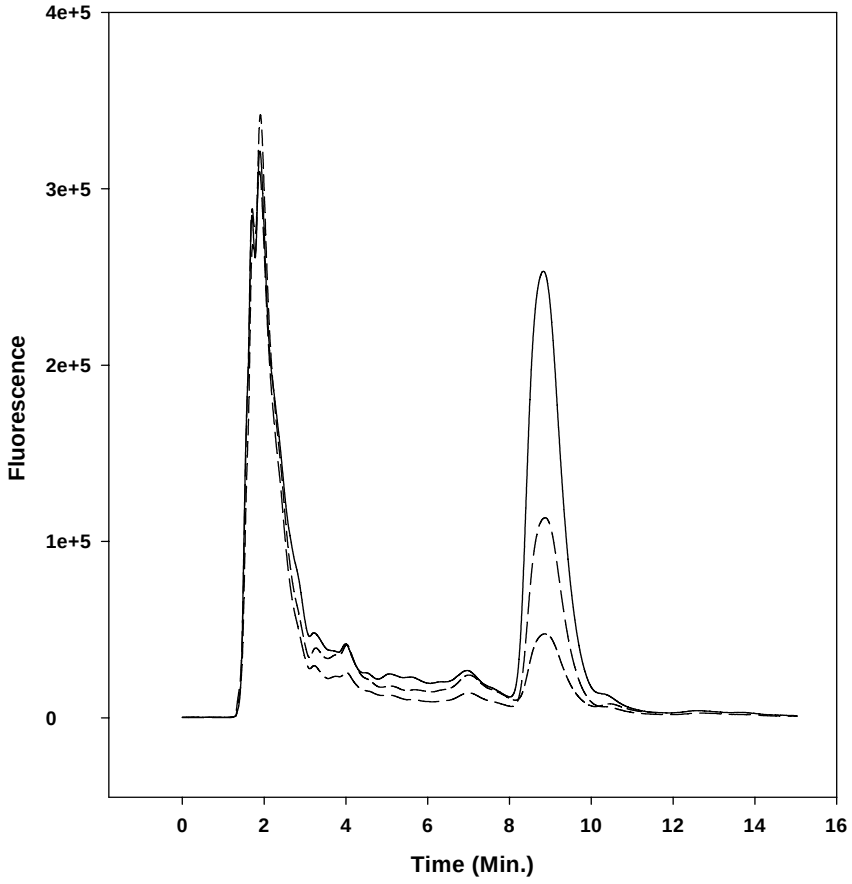


Figure 2.23 Chromatograms indicating II formation in the standard Fenton reaction (——), standard Fenton reaction with Fe (III) (— ——) and a blank reaction with Fe (III) but without Fe (II) (- - -), in presence of methane and 3ap. Standard reaction conditions; 50 μM 3ap; 1.5 mM CH_4; 150 μM H_2O_2; 10 μM Fe (II); 10 mM Fe(III) in 2 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents and allowed to proceed to completion (15 minutes).

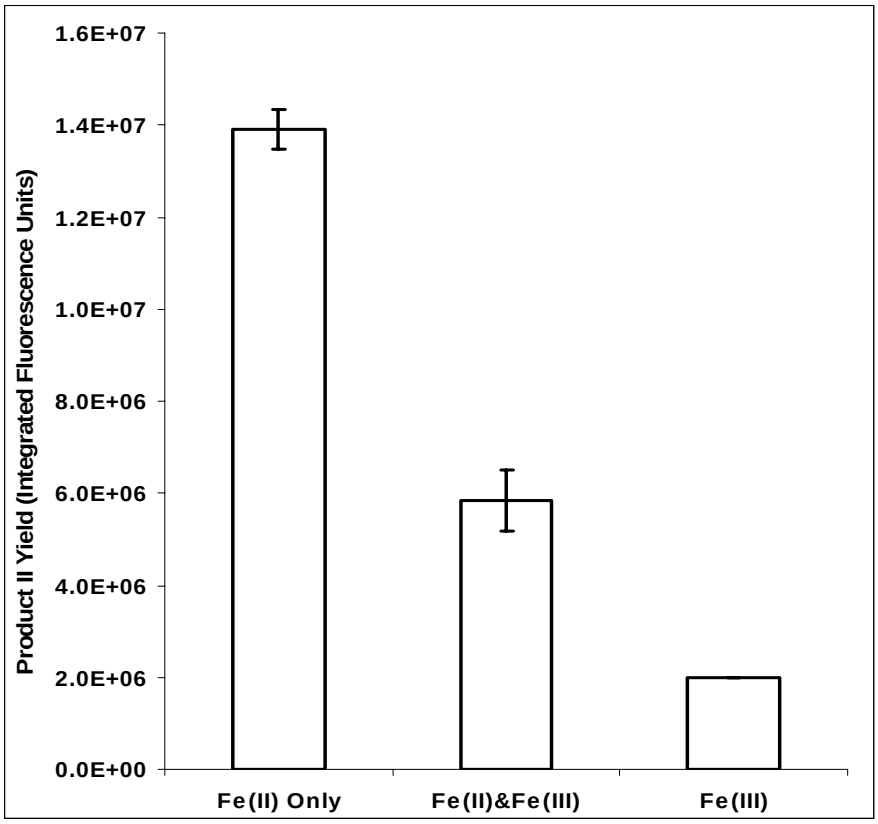


Figure 2.24. II yields in a standard Fenton reaction with and without 10 mM Fe (III) as well as in a blank reaction containing only 10 mM Fe (III) without Fe (II) in presence of methane and 3ap. Standard reaction conditions; 10 μM Fe (II), 50 μM 3ap, 10 mM Fe (III); 150 μM H_2O_2 in 2 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) into an anaerobic reaction mixture containing all other reagents and allowed to proceed to completion (15 minutes). The error bars represent standard deviation obtained from a total of three experiments in each case.

2.4 Summary & Conclusion

2.4.1 Summary

From the foregoing results, it is evident that Fenton reactions in which the Fe (II) is coordinated with an inorganic ligand (phosphate), produce an intermediate oxidizing species indistinguishable from HO. The evidence supporting HO involvement in Fenton reactions with the inorganic Fe complexes includes:

(i) The ratio of the rate constants $R = k_{3ap}/k_{DMSO}$, obtained from the nitrate photolysis experiment, (a well known source of HO), is identical to the literature value expected for HO from pulse radiolysis studies (i.e. $R = 0.74 \pm 0.09$), and compares very closely to the values obtained in the Fenton reaction at both acidic and neutral pH, (Table 2.2).

(ii) Product comparison studies of the Fenton reaction at pH 4.2 and 7.4 yield Y_{DMSO}/Y_{CH4}, values comparable to the expected literature values for HO from pulse radiolysis studies, (Table 2.2, Column 2).

(iii) In competition in the presence of either DMSO or CH_4 at both acidic and neutral pH, the experimental rate constant for the reaction of species involved with BA (k_{BA}) yields k_{BA} values, very close to the literature value for the reaction of BA with HO, (Table 2.3).

(iv) Studies of the Fenton reaction in presence of large excess of Fe (III), in the presence of 3ap and DMSO, show minimal change in II yield associated with HO generation. However, the same reaction in presence of methane shows a significant reduction in II formation. These results indicate that the oxidizing species formed in the reaction of Fe (III) with HO (e.g. ferryl species) oxidize DMSO to yield methyl radicals resulting in minimal change in the total II yield. In the case of methane however, the ferryl species is not able to oxidize methane, and therefore the lower II yield obtained is only from the oxidation of methane by HO.

2.4.2 Conclusion

These studies indicate that the oxidizing intermediate(s) produced in the Fenton reaction reacts with both DMSO and methane to yield methyl radicals. Comparison of the product yields for the DMSO and methane experiments performed under identical conditions at both the acidic and neutral pH, show that the species involved is HO. Similarly, competition studies with the nitroxide and benzoic acid also show HO involvement under all the conditions of this study.

The extent of HO involvement as evidenced by the amount of II formed is found to be 90 % at acidic pH and to decrease substantially (~20 %) at neutral pH. This reduction may result from trace contamination of oxygen oxidation of Fe (II) at high pH, thus reducing the amount of metal available for the Fenton reaction.

Studies with Fe (III) show that the species formed in the reaction of Fe (III) and HO are capable of oxidizing DMSO to yield methyl radicals but cannot oxidize methane.

CHAPTER III

Fenton Reactions with Organic Iron Complexes

3.1 Introduction

Considerable debate on the nature of intermediates produced in the Fenton reaction with chelated Fe (II) exists. The organic ligands are commonly used to form water-soluble complexes with multivalent metal ions. The purpose of the chelating agents in this case being to bind with the metal ions in such a way as to prevent undesirable interactions.

Of the three ligands employed in this study, EDTA, NTA and DTPA (Figure 1.1, chapter I), EDTA has been the most widely studied. Its attractiveness stems from its well understood stoichiometric reaction with several metal ions and from its ability to react with most metals to form complexes with high binding constants. Though several studies have concluded that free OH may be the species involved when iron is chelated to this ligand (Cheves et al 1970, Halliwell et al 1978, Buettner et al 1983, Luzzato et al 1995) a good number of recent studies question this conclusion and instead invoke high-valent metal species (Rush and Koppenol 1986, Welch et al 2002). Yet another group of studies suggest the possible involvement of both species (Yamazaki et al 1992, Winterbourne 1987) and that the distinction between these species may be difficult (Rao et al 1996) or may largely depend on the conditions of the study (Meyerstein 1999).

In the case of Fe (II)-NTA, a study by Rush and Koppenol (1988), lays the foundation for the possible involvement of high valent species. However, several other

key studies (Czapski et a, 1988; Hamazaki et a, 1989; Aruoma et al 1989, and Iqbal et al, 2003) favor involvement of OH in Fenton reactions involving this ligand.

Studies with Fe (II)-DTPA have not been spared of the confusion, though a number of these studies have clearly pointed to the formation of HO in Fenton reactions in the presence of this ligand (Egan et al, 1992; Kikalor et al, 1998); other studies have indicated possible involvement of free OH, high-valent metal species, or both (Yamazaki 1991, 1992).

For proponents of free OH, the rate of OH generation in the chemical system has been found to depend on the type of chelator used and the concentration of the metal complex involved. This rate has been found to be relatively fast for EDTA compared to NTA or DTPA. An increase in the number of bonds between the ligand molecules and ferrous iron enhances OH production, in the order NTA < EDTA < DTPA supporting the steric origin for this enhancement. The time of reaction is also found to correlate with the charge on the ferrous iron complex i.e. NTA < EDTA < DPTA which have a negative charge of one, two and three respectively (Kachur et al 1998).

From the foregoing statements, it is apparent that the nature of intermediates produced in the Fenton reaction by Fe coordinated to these organic ligands is still a matter of considerable debate. With this in mind, this study was carried out with the objectives of determining the nature of the intermediates produced in Fenton reaction in the presence of organic ligands especially at neutral pH, the efficiency of production of these species by the various ligand systems, and factors such as pH and speciation that may govern production of the oxidizing intermediate(s).

In order to achieve these objectives, the Fenton reaction was carried out with organic metal complexes of iron under conditions similar to those described in the previous chapter. In this case, experiments were performed in presence of DMSO, methane, and nitroxide (3ap). The yields of product II in these experiments (Scheme 1.5), was evaluated to test if they conformed to those expected for HO. The dependency of the yield of II on the concentration of the Fe complexes was measured to determine the stoichiometry of the reaction.

3.2 Experimental

3.2.1 Apparatus

The same HPLC set up described in chapter II was used for the separation and detection of II. The chromatographic conditions were identical to those used in the case of the phosphate complexes, described previously (Section 2.2.1).

3.2.2 Materials

Ethylenediamine tetraacetic acid di-soduim salt (EDTA, $C_{10}H_{14}N_2Na_2O_8.2H_2$, 99% purity), and nitrilotriacetic acid (NTA, $C_6H_7NO_6Na_2$) di-sodium salts (99% purity) were obtained from Sigma. Diethylenetriamine pentaacetic acid (DETAPAC, $C_4H_{23}N_3O_{10}$, 99% pure) was obtained from Fluka. Other materials and chemicals used in this study were identical to those used in chapter II and were obtained from the same sources, (Section 2.2.2).

3.2.3 Experimental preparations

Stock solutions of the metal chelate complexes ranging in concentration from 10 - 25 mM were prepared by dissolving a known amount of the pure ligand in water or 2 mM phosphate buffer at pH 4.2 or 7.4. Lower stock concentrations (400 μM) of the ferrous complexes were then prepared daily by dissolving appropriate amounts of the metal ions with the ligands in a 1:1.3 ratio in water or buffer. The stock solutions of the metal complexes were purged with N_2 throughout the day. Other reagents for use in these studies were prepared as described elsewhere (Section 2.2.3).

3.2.4 Experiment Protocol

3.2.4.1 Experiments with DMSO

To determine whether the oxidizing intermediate formed in the Fenton reaction with organic Fe complexes, are able to oxidize DMSO, and yield II, 10 mM DMSO and 50 μM 3ap were added to a solution of phosphate buffer at pH 4.2 or 7.4 in a 4 mL cuvette. The resulting solution was then N_2 purged for 5 minutes before 150 μM H_2O_2 (N_2 purged) was added via a gas-tight syringe. The reaction was initiated by addition of 10 μM iron complex (using a gas-tight syringe) from a N_2 purged stock solution of the metal complex.

The reaction was allowed to continue under nitrogen for 15 minutes before the pH was raised and the solution derivatized with fluorescamine with the resulting products then separated and quantified on the HPLC as described in Section 2.2.4.1 (Chapter II). In competition experiments with the nitroxide, lower, fixed concentrations of DMSO, 150 μM or 300 μM were used in reactions while the nitroxide, concentrations were varied from 0.15 mM to 2.4 mM.

3.2.4.2 Experiments with methane

To determine whether the oxidizing intermediate formed in the Fenton reaction with organic Fe complexes, are able to oxidize methane, and yield II, 1.5 mM CH_4 and 50 μM 3ap were added to a solution of phosphate buffer at pH 4.2 or 7.4 in a 4 mL cuvette. The resulting solution was then purged with methane for 10 minutes before 150 μM H_2O_2 (N_2 purged) was added via a gas-tight syringe. The reaction was initiated by addition of

10 μM iron complex (using a gas-tight syringe) from a N_2 purged stock solution of the metal complex.

The reaction was allowed to continue under methane for 15 minutes before the pH was raised and the solution derivatized with fluorescamine with the resulting products then separated and quantified on the HPLC as described in Section 2.2.4.1 (Chapter II).

3.2.4.3 Experiments at Varying concentrations of the iron ion complex

The dependence of the yield of II on Fe complex concentration was determined over the range of 5 μM to 25 μM of the metal complex. In the experiments, 10 mM DMSO, and 50 μM 3ap, (in 2 mM phosphate buffer at pH 4.2 or 7.4) were nitrogen purged for 5 minutes before addition of 150 μM H_2O_2 (N_2 purged) using an air-tight syringe. The reaction was then initiated by addition of 10 μM of Fe complex.

3.2.4.4 Experiments at Varying [H_2O_2]

Experiments were carried out at varying concentrations of excess hydrogen peroxide in a bid to determine whether O_2 contamination was causing partial oxidation of Fe (II), thus decreasing the yield of II in the Fenton reactions at neutral pH. In these experiments, the concentration of hydrogen peroxide was doubled and tripled so as to compete with any contaminating O_2 in the reaction. In a series of these experiments, varying concentrations of H_2O_2, ranging from 150 μM - 500 μM were added to a mixture of 50 μM 3ap and 10 mM DMSO, in 2 mM phosphate buffer at pH 7.4. The reactions were then initiated by addition of 10 μM of the ferrous metal complex and allowed to

continue to completion (for 15 minutes) before the reaction was derivatized, separated and quantified as described in Section 2.2.4.1(Chapter II).

3.3 Results and Discussion

3.3.1 Fenton Reaction: DMSO experiments

When the Fenton reaction was carried out with the ferrous metal chelated to either DTPA, EDTA, or NTA, in presence of dimethyl sulfoxide and 3ap, significant yields of II were obtained (see Figure 3.1, for the Fe-DTPA complex). II was not formed in the absence of the metal complexes or DMSO. These results indicated that the oxidizing intermediate produced in the Fenton reaction with organic complexes were capable of yielding methyl radicals via reaction with DMSO.

As a further test, competition experiments were performed with 3ap in the presence of DMSO. When the concentration of 3ap was increased appropriately so as to compete with a fixed amount of DMSO, II yield was found to decrease substantially (Figure 3. 2). The decrease in II yield was found to follow a non-linear regression of the form $f_n = a/ (1 + bx)$, which conforms to the (expression II-1), employed earlier (Section 2.3.1).

For the Fe(II)-DTPA complex, the experimental ratio of $k_{3ap}/k_{DMSO} = 1.02 \pm 0.24$, obtained from a fit of the experimental data to expression II.1, agrees with the expected value of $k_{3ap}/k_{DMSO} = 0.74 \pm 0.09$, obtained in the case of nitrate experiment, a well known free HO source. Similar results were obtained in studies with the other metal complexes (Fe (II)-EDTA, Figure 2.3 and Fe-NTA, Figures 2.4 and Figure A10,

Appendix). These findings (Table 3.1) clearly indicate that HO is involved in Fenton reactions with these organic metal complexes.

Table 3.1 List of findings for ligand studies

Metal Complex	**k_{3ap}/k_{DMSO} = b*[DMSO]**
Fe-DTPA	1.02 ± 0.24
Fe-EDTA	1.07 ± 0.35
Fe-NTA	0.78 ± 0.05 0.65 ± 0.05
Nitrate Experiment value	0.74 ± 0.09

* b values obtained from least squares fit of experimental values to Expression II.1, (Figures 3.2, 3.3, and 3.4) and are contained in Table A1 (Appendix). Literature value obtained from known rate constant of the reaction of HO with DMSO and 3ap, (Table B2, Appendix).

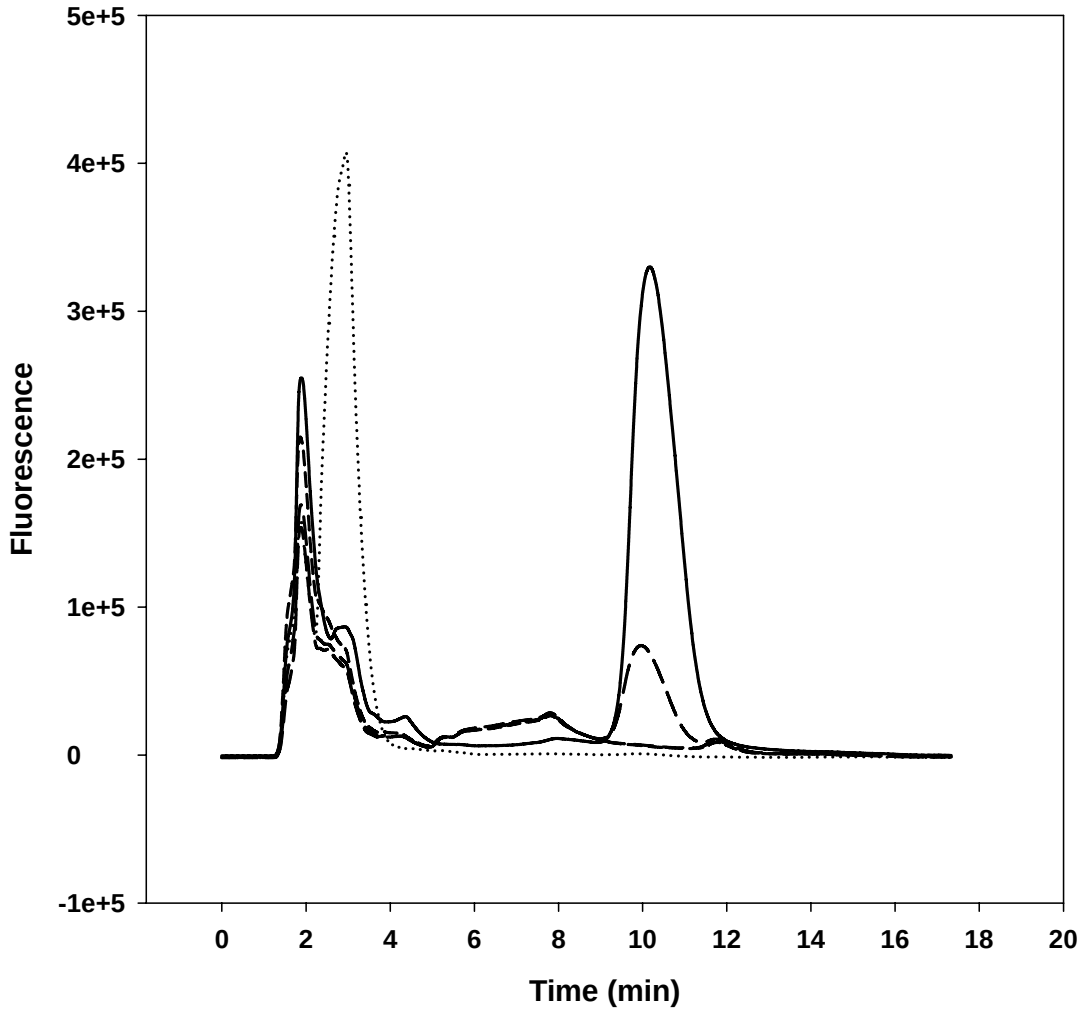


Figure 3.1. Chromatograms illustrating II formation with Fe (II)-DTPA in presence of DMSO under anaerobic conditions. Standard reaction conditions; [3ap] = 50 μM, [DMSO] = 1.5 mM, [H_2O_2] = 150 μM, in 2 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 μM Fe (II)-DTPA and allowed to continue for 15 minutes. In this case, standard reaction (——), standard reactions with Fe phosphate in absence of the DTPA ligand (—— ——), standard reaction without Fe (II)-DTPA complex (— — —), standard reaction without DMSO (-----) and standard reaction without H_2O_2 (.....).

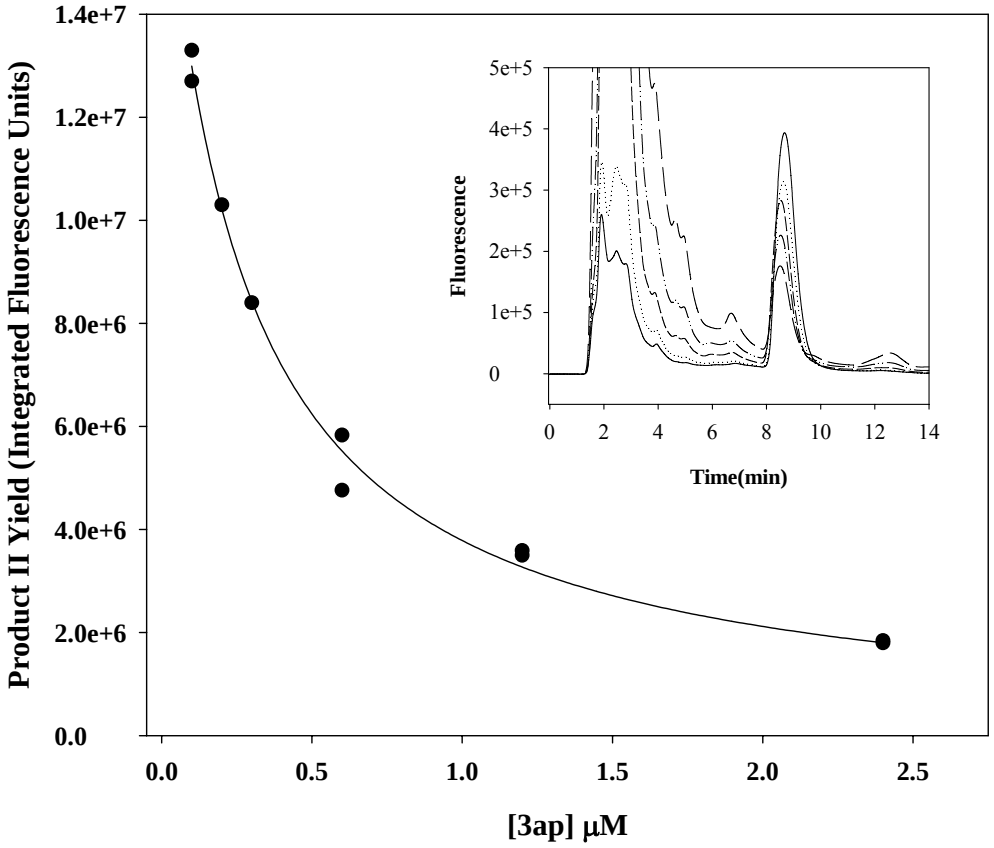


Figure 3.2. The dependence of II yield on 3ap in reaction with Fe (II)-DTPA in presence of a fixed concentration of DMSO. Reaction conditions; 300 μM DMSO, 10 μM Fe (II) - DTPA, 150 μM H_2O_2, in 2 mM phosphate buffer at pH 7.4. The reaction was initiated by addition of Fe (II)-DTPA and allowed to continue anaerobically for 15 minutes. The curve represents a least squares fit to the form $f_n = a/ 1 + bx$.

Inset, chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. Inset, chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. The experiments were performed with varying amounts of 3ap; (——) 0.10 mM, (.....) 0.30 mM, (— — —) 0.60, and (— · · —) 1.2 mM and (—— ——) 2.4 mM respectively.

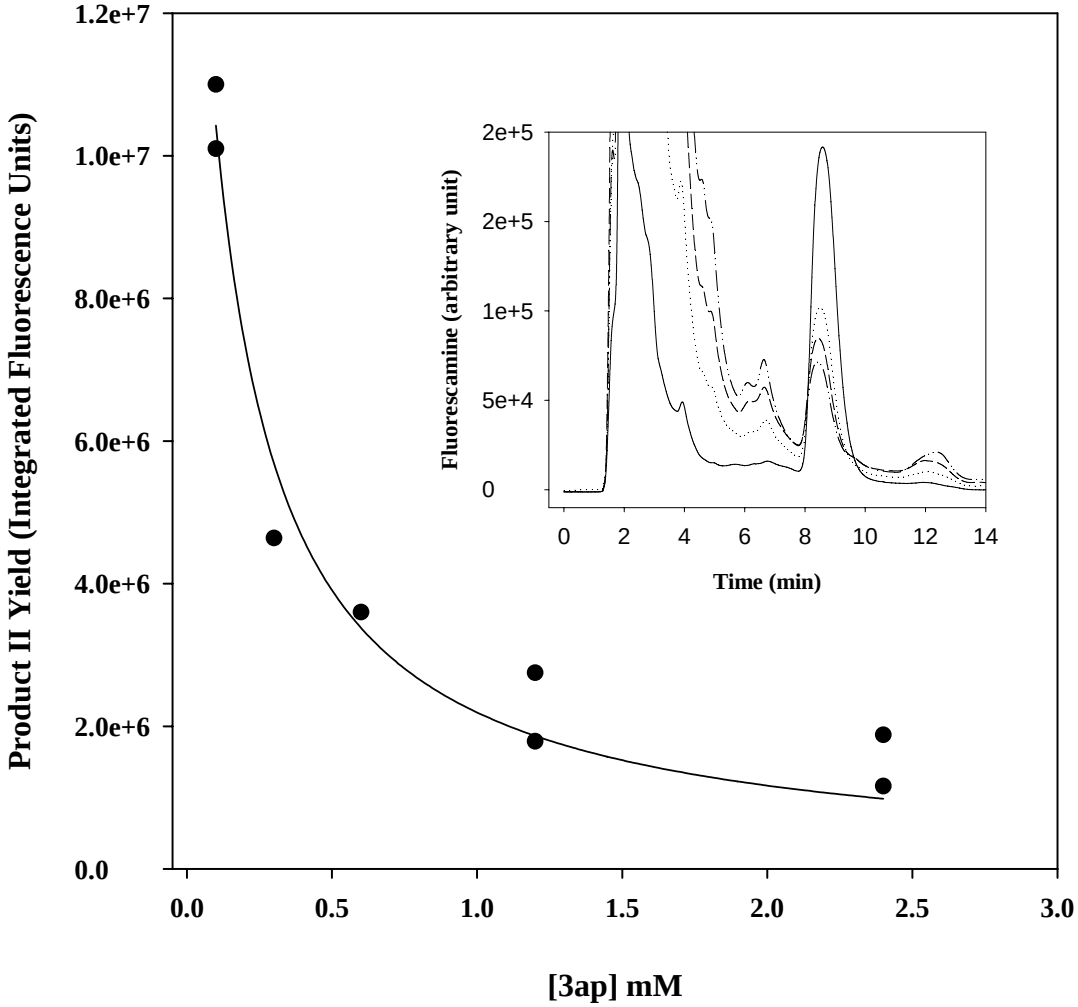


Figure 3.3. The dependence of II yield on 3ap in reaction with Fe (II)-EDTA in presence of a fixed concentration of DMSO. Reaction conditions; 150 μM DMSO, 10 μM Fe (II)-EDTA, 150 μM H_2O_2, in 2 mM phosphate buffer at pH 7.4. The reaction was initiated by addition of Fe (II)-EDTA and allowed to continue anaerobically for 15 minutes. The curve represents a least squares fit to the form $f_n = a/ 1 + bx$.

Inset, chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. The experiments were performed with varying amounts of 3ap; (——) 0.10 mM, (.....) 0.60 mM, (– – –), 1.2 mM and (— · · —) 2.4 mM.

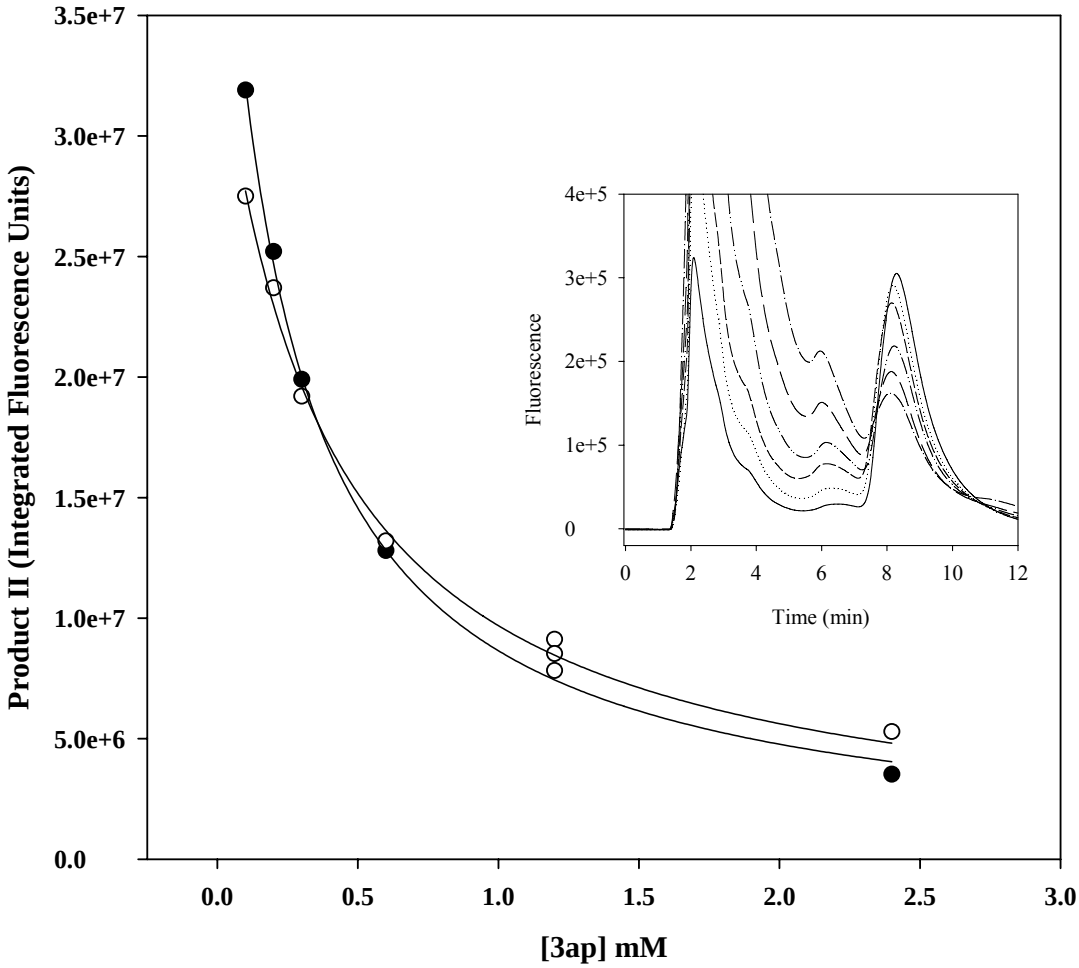


Figure 3.4. The dependence of II yield on [3ap] in the reaction of Fe (II)-NTA with H_2O_2 in presence of a fixed concentration of DMSO. Reaction conditions: 10 µM Fe (II)-NTA; 150 µM H_2O_2, in 2 mM phosphate buffer at pH 7.4, with (●) 300 µM and (○) 150 µM DMSO. The reaction was initiated by addition of Fe-NTA and allowed to continue anaerobically for 15 minutes. The curve represents a lest squares fit to the form $f_n = a/ (1 + bx)$. Inset, chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. The experiments were performed with varying amounts of 3ap; (——) 0.10 mM, (.....) 0.20 mM, (– – –) 0.30, and (— · · —) 0.6 mM and (—— ——) 2.4 mM respectively.

3.3.2 Fenton reactions: methane experiments

As previously stated, Fenton reactions were carried out in methane in place of DMSO to test whether the oxidizing intermediates involved under these conditions would oxidize methane to yield II. When Fenton reactions with Fe-DTPA were performed in presence of methane, large amounts of II were obtained which were not observed in the absence of methane or metal complex indicating that a sufficiently strong oxidizing intermediate most likely HO is involved in the Fenton reactions with these organic metal complexes.

As a further test of the nature of the species involved, II yield in the DMSO experiment was compared to that in the methane experiment, done under identical conditions. In reaction conditions, where the concentration of DMSO is saturating, expression II.10, representing the ratio of II yield in the DMSO and methane experiments may be used.

For the Fe (II)-EDTA complex, the experimental values of 3.4 ± 0.7, and 3.34 ± 0.3 obtained for the reactions at acidic and neutral pH compares closely with the expected literature value of 3.0, obtained from the known rate constants of the reaction of HO with 3ap, DMSO and CH_4. Experimental values that were very close to the literature values were also obtained for the other metal complexes, Fe-DTPA and Fe-NTA (Table 3.2), further indicating the involvement of HO in these reactions.

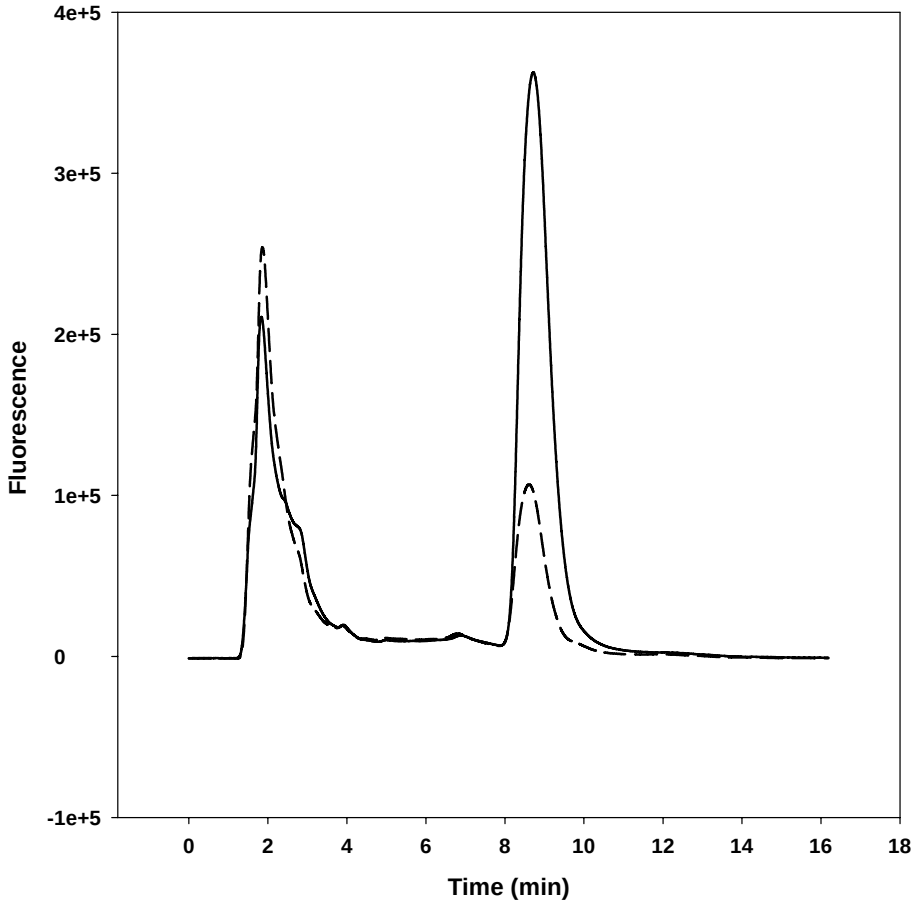


Figure 3.5. Chromatograms indicating HO trapping with (1.5 mM) CH_4 and DMSO (10 mM) under anaerobic conditions. Standard reaction conditions; 10 μM Fe (II)-DTPA, 50 μM 3ap, 150 μM H_2O_2 in 2 mM phosphate buffer at pH 4.2. In this reaction, standard reaction with DMSO (——), standard reaction with methane (- - -). The reactions were initiated by addition of 10 μM Fe (II) - DTPA into an anaerobic reaction mixture containing all other reagents. The reaction was then allowed to continue to completion (for 15 minutes) before it was derivatized and injected onto the HPLC.

Table 3.2 Product comparisons Y_{DMSO}/Y_{CH4}

Metal Complex	Y_{DMSO} / Y_{CH4} [1]	
	pH 4.2	pH 7.4
Fe-EDTA	3.4 ± 0.7	3.3 ± 0.3
Fe-DTPA	3.6 ± 0.4	3.5 ± 0.5
Fe-NTA	3.0 ± 0.3	3.5 ± 0.5
Literature value	3.0	3.0

[1] Standard error obtained from an average of three values obtained from three independent experimental studies (Table A3, Appendix).

3.4.3 Dependence of II yields on [Fe(II)]

Results from the preceding sections so far, give very strong indication of the involvement of HO in Fenton reactions employing organic ligands. In order to determine the extent of involvement of HO under these conditions, and obtain an insight into the reaction stoichiometry for the Fenton reactions involving the organic metal complexes, the Fenton reactions were carried out with varying concentrations of the metal complex at fixed concentration of the nitroxide and DMSO.

When the concentration of the Fe complexes was increased, II formation increased accordingly, (Figure 3.6). This increase which was observed for all the organic

metal complexes studied at acidic and neutral pH (Figures 3.7 and 3.8), was found to correspond to relatively higher yields of II at lower pH 4.2 (Figure 3.7) compared to those obtained in at neutral pH (Figure 3.8).

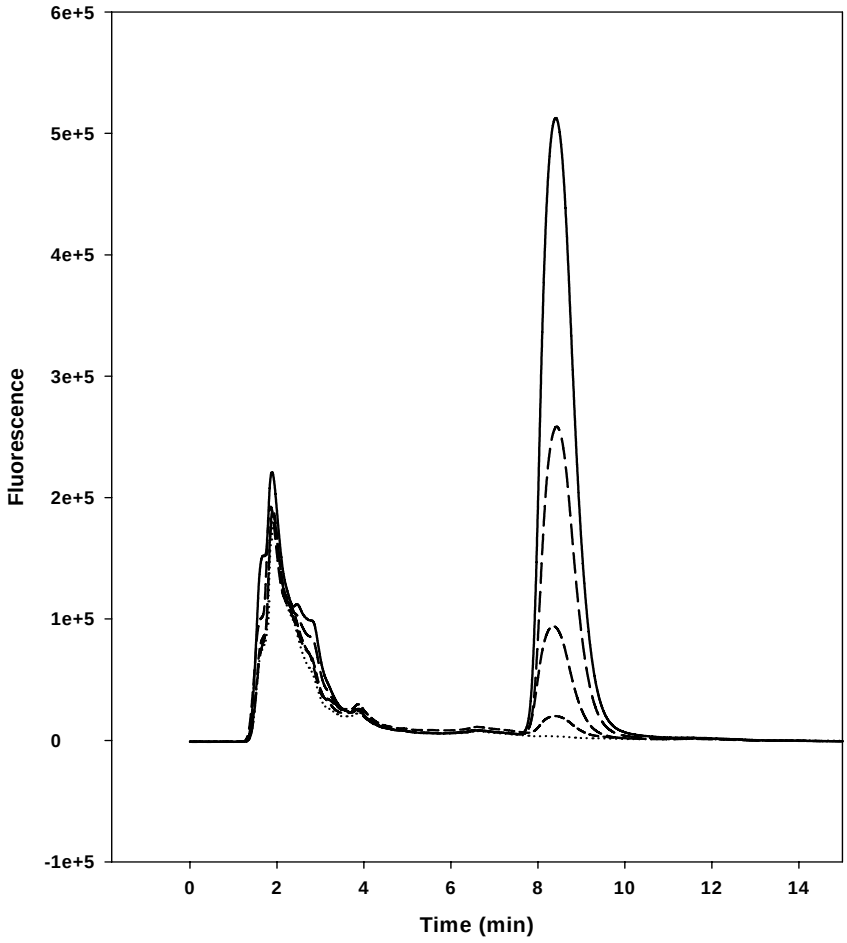


Figure 3.6. Chromatograms showing dependence of the yield of II on Fe (II)-EDTA concentration. Reaction conditions; [DMSO] = 10 mM, [3ap] = 50 μM, [H_2O_2] = 150 μM, in 2 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 μM Fe (II) and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of Fe (II)-EDTA; 25.0 μM, (——); 17.0 μM, (— —); 10.0 μM, (— — —) and 5.0 μM, (-----), and 0 μM (.....), Fe (II)-EDTA.

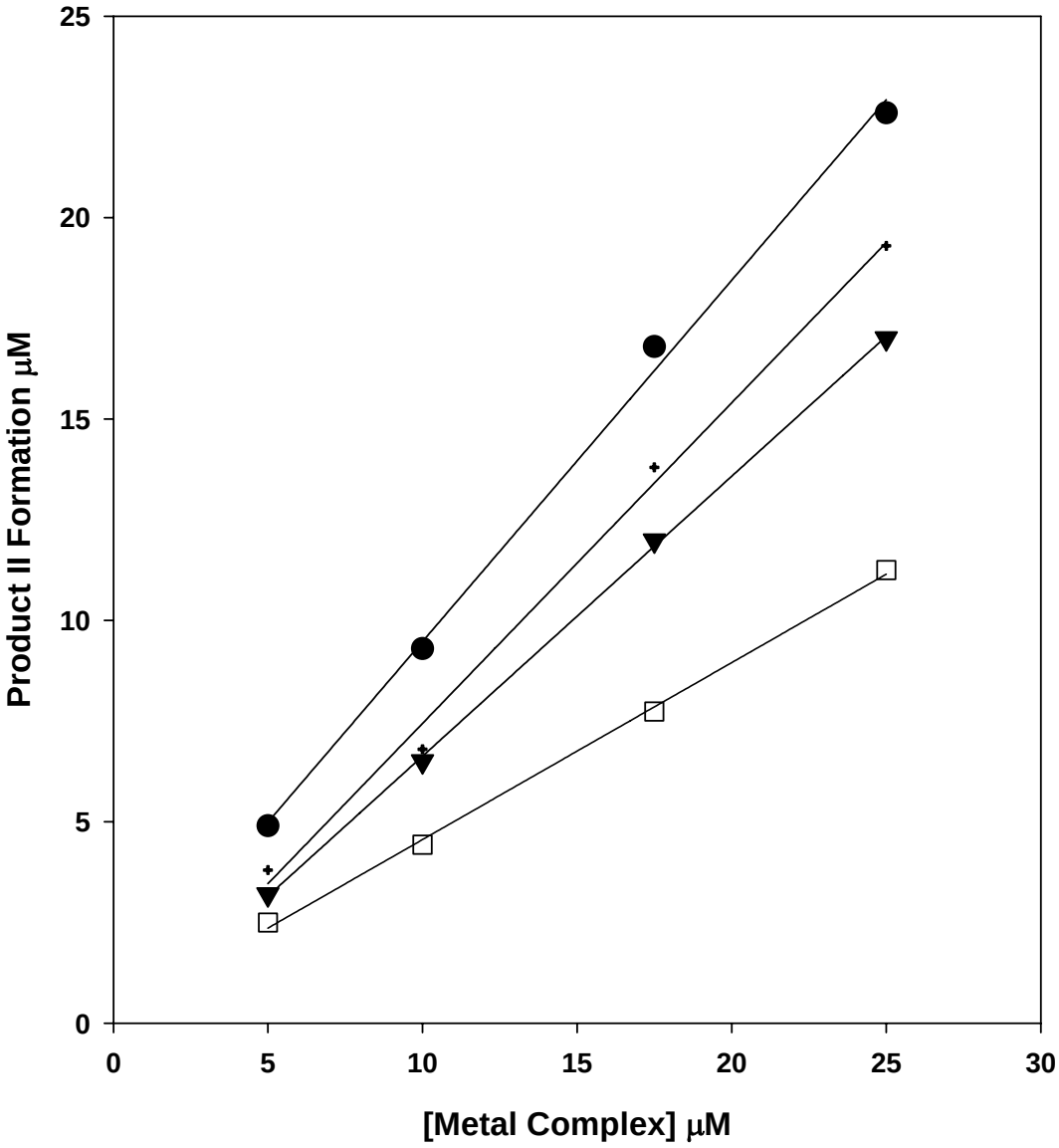


Figure 3.7. II formation with increasing concentration of metal ion. Reaction conditions; [DMSO] = 10 mM, [3ap] = 50 μM, [H_2O_2] = 150 μM, in 2 mM phosphate buffer at pH 4.2. Reaction was initiated by addition of the metal ions of varying concentration. The reactions were performed using various ferrous iron complexes; Fe -phosphate, (●); Fe-EDTA, (+); Fe-DTPA, (▼) and Fe-NTA, (□).

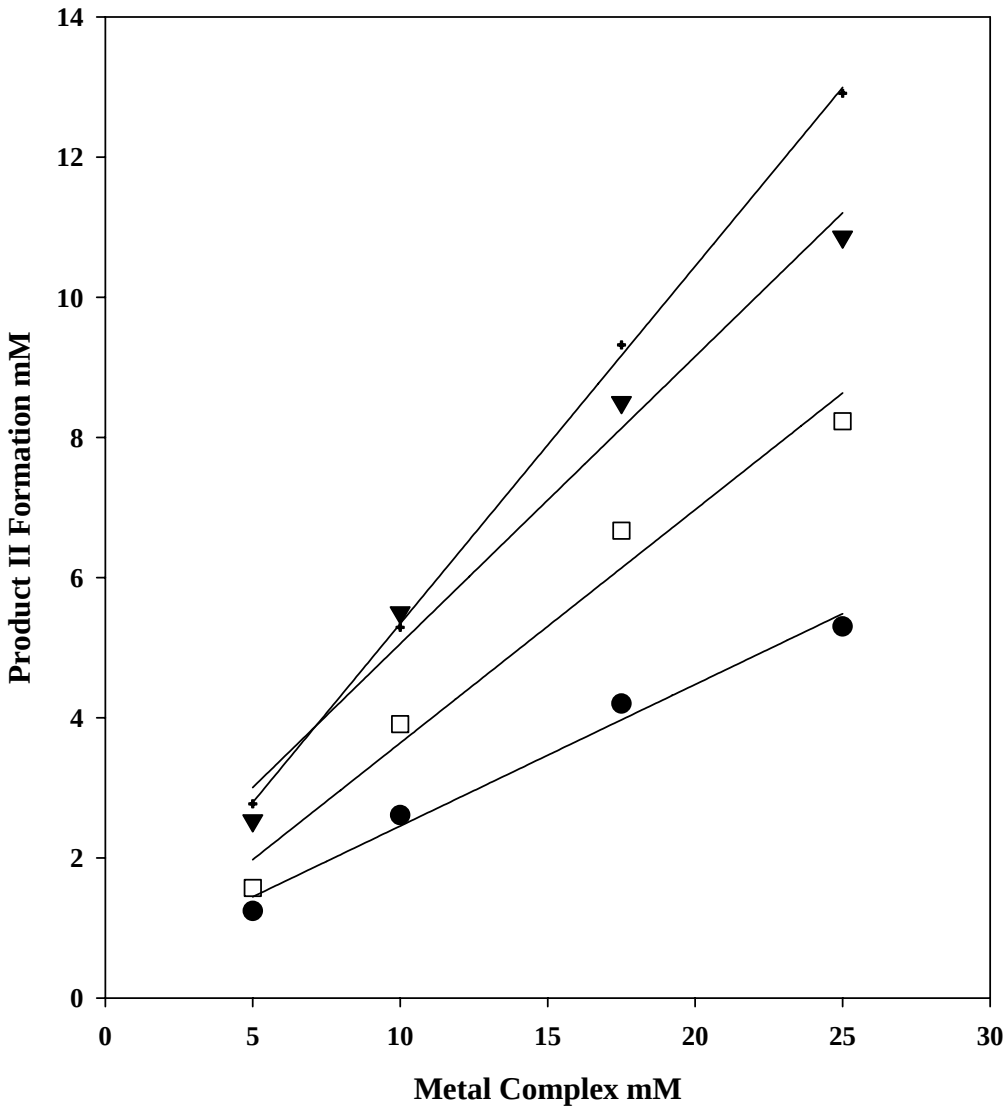
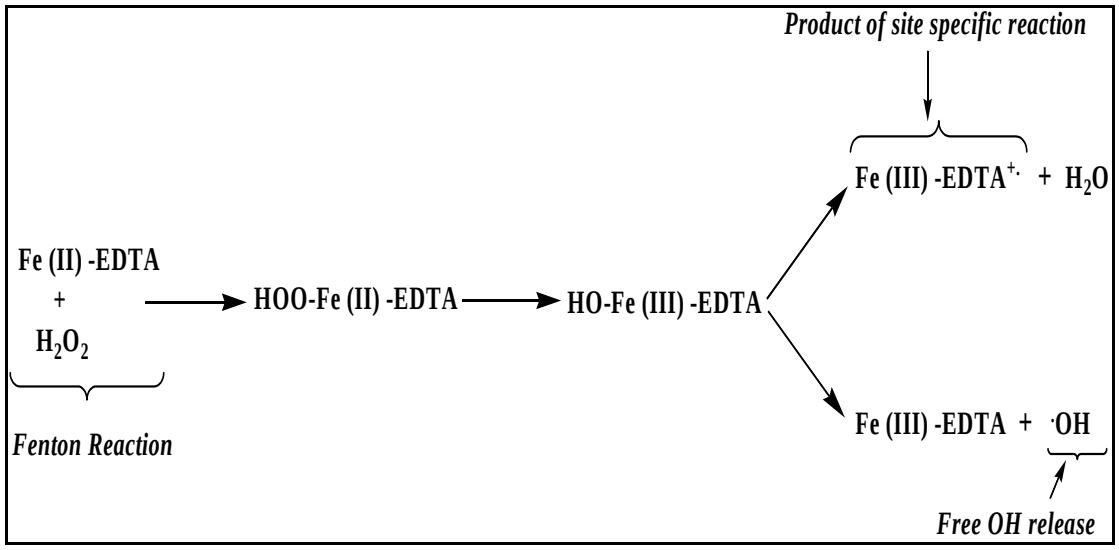


Figure 3.8. II formation with increasing concentration of metal ion. Reaction conditions; [DMSO] = 10 mM, [3ap] = 50 μM, [H_2O_2] = 150 μM, in 2 mM phosphate buffer at pH 7.4. Reaction was initiated by addition of the metal ions of varying concentration. The reactions were performed using various ferrous iron complexes; Fe-DTPA, (+); Fe-EDTA, (▼) and Fe-NTA, (□). The Fe-phosphate reaction (●) was performed in 100 mM phosphate buffer at pH 7.4.

This reduction in product yield in presence of organic ligands may be attributed to a site specific reaction where the free radicals are formed in close proximity to the organic ligand. The radicals then, preferentially reacts with the ligand complex near the site of formation before being released to the solution to be scavenged by the primary HO scavenger such as DMSO (Scheme 2.1). Similar conclusions have been made in other studies (Samuni et al 1983). Such a reaction would result in a reduction in the amount of HO scavenged by the primary HO scavenger, leading to reduced yields in methyl radicals and eventually the amount of II observed. This competitive reaction of the organic ligands is thought to be even more likely to occur, considering the high values of the rate constants of the reaction of HO, with the organic ligands, (Table 3.4) which are somewhat comparable to its reaction with DMSO.



Scheme 3.1 Site Specific reaction with organic metal complexes

Table 3.4 Rate constants for the reaction of HO with Organic Ligands

Ligand	pH	K ($x10^9$, $M^{-1}s^{-1}$)
DTPA	5 - 11	5
EDTA	4, 9	0.4, 0.2
NTA	0 - 9	2.3

The yield of II at lower pH is found to be lower in the case of (Fe(II)-DTPA compared to Fe (II)-EDTA and this may be partly due to several factors including; the high rate of reaction of HO with Fe (II)-DTPA compared to its reaction with Fe(II)-EDTA (Table 3.5). Therefore, the site specific reaction between HO with the Fe (II)-DTPA complex occurs more readily in the case of Fe (II)-DTPA compared to Fe-(II) EDTA. Additionally, it may be attributed to the differences in the reaction of the metal complexes with oxygen and hydrogen peroxide (Table 3.3 below), where the reaction with peroxide may be much lower in the case of the Fe (II)-DTPA complex compared to that with the Fe (II)-EDTA metal complex.

Table 3.5 II formation with rate constants for the reaction of iron metal complexes with O_2 and H_2O_2

Fe-II Metal Complex	% Product Formed *		Rate Constant for the Reaction with dioxygen, $k/M^{-1}s^{-1}$		Rate Constant for the Reaction with H_2O_2, $k/M^{-1}s^{-1}$	
	pH 4.2	pH 7.4	pH 4.2	pH 7.4	pH 4.2	pH 7.4
Fe-Phosphate	90 ± 5	20	52	1×10^3		$1 \times 10^3 - 2 \times 10^4$
Fe-NTA	42	33				
Fe-EDTA	79 ± 1	41 ± 1	63	100	7.2×10^3	1.4×10^4
Fe-DTPA	65 ± 6	53 ± 3	13.5	3.5		4.1×10^3

* The percentage product formed and standard errors were based on the slopes in Figures 3.7 and 3.8, and the percentage product formed was based on the initial concentration of the Fe-L complex. Rate constant values were obtained from various references (Table B1, Appendix B).

On the other hand, II formation at high pH for all the ligands is much higher than that in the case of the Fe phosphate complex. This may be due to the ability of the organic ligands to strongly bind the metal ions and form soluble metal ligand complexes, while blocking undesirable interactions associated with free metal ions. This scenario, which may not be true for the phosphate metal complexes, renders the organic ligand complexed metal ions more available for the Fenton reaction. The low yield of II at neutral pH may also be due the possible involvement of oxidizing species other than HO.

In an attempt to determine the oxygen contamination at high pH, Fenton reactions were performed at high concentrations of hydrogen peroxide so as to out compete the oxygen for the ferrous ions. However, there was minimal change in the yield of II under the varying concentrations of peroxide (Table 3.6), indicating that the oxygen contamination was immeasurable at least with this methodology.

Table 3.6 II formation at Varying [H_2O_2] at pH 7.4

[H_2O_2] µM	II Formation µM	
	EDTA	**Phosphate**
125	5.8	2.61
150	5.6	2.60
500	5.3	2.07

3.5 Conclusion

Fenton reaction studies with polyaminocarboxylic acid complexes of iron, EDTA, DTPA, and NTA, show involvement of oxidizing intermediates capable of yielding methyl radicals with both DMSO and methane. Furthermore, product yields and distribution associated with these intermediates species typify and confirm them to be HO. The studies find HO involvement to be manifested at both acidic and neutral pH for all the ligand systems studied. The stoichiometric yield of HO is found to decrease with increasing pH possibly due to several factors including; possible competitive oxidation of the iron (II) by contaminating oxygen at higher pH (especially for the phosphate metal complex), possible oxidation of the ligand in a site-specific reaction by HO (as has been suggested by others), as well as the possible involvement of other oxidizing intermediate species depending on the metal complex.

CHAPTER IV

Conclusions and Future Work

4.1 Conclusions

The objective of this work was to determine the nature of the species involved in Fenton reactions at various conditions and how these factors such as pH and the presence of ligands influence the production of these species. These objectives were met; this study shows HO involved in all conditions of the study i.e. at mildly acidic and neutral pH and in presence of ligands.

Whereas earlier work suggested that there is no HO or free radical involvement in Fenton or Fenton –like reactions (Sawyer et al 1996, Kremer 2002, Dunfold 1999), this study has clearly shown HO to be generated in Fenton reactions involving organic and inorganic ferrous complexes. Earlier studies also claimed that HO was probably involved only at lower pH (Hug et al), but this study has shown that HO is involved at both low and neutral pH. Furthermore, earlier studies by Sawyer (1996) and Kremer (2002) claimed that no one has been able to get Fenton and Fenton-like reagents to react with methane; I show for the first time that Fenton and Fenton-like reagents can oxidize methane to yield methyl radicals.

Similarly, though many previous studies have pointed out the difficulty of distinguishing between the hydroxyl radical and high-valent metal species, I provide a simple method to distinguish between the two species by reaction with methane and DMSO employing a sensitive detection technique. Most of the earlier studies done to

determine the nature of species involved in Fenton and Fenton-like reactions have been found to be inconclusive, due to the limitations and artifacts in their methodology. This systematic study, which employs new approaches, has provided clear evidence of HO involvement in the Fenton and reactions.

Distinctively, this study due to its systematic approach and use of several techniques (methods), such as the use of HO scavengers DMSO and methane, and introduction of HO competing substrates such as 3ap and BA, surpasses previous studies. This is because in all cases this study yields information that is consistent with free HO, as compared to literature values and values obtained from an independent free HO source, (nitrate photolysis). The study therefore provides a more conclusive proof of free HO involvement in Fenton reactions with inorganic and organic complexes of iron.

4.2.1 Future Work

Although key objectives of this study were met, further work still needs to be done in regard to some areas. Although HO was found to be involved in Fenton reactions with organic metal complexes, yields of II associated with HO production for these metal complexes at lower pH were lower than those in the case of inorganic ligands. Redox cycling experiments involving ferric organic metal ligands need to be done with a suitable metal reductant so as to determine the HO yield under catalytic conditions. It is expected that such a study might cause the ligand to be destroyed, which will be reflected in the yield of II.

Fenton experiments at neutral pH gave much lower yields of II, although this was attributed to the competing side reactions such as oxidation by oxygen, and differences in

the rate of Fenton reaction with the various metal ligands. Further experiments need to be done to ascertain this. Experiments monitoring the amount of Fe (II) or Fe (III) during and at the end of the reactions (e.g. spectrophotometrically) may provide further insight into the reaction stoichiometry and extent of oxygen contamination.

Although experiments with Fe(III) provided a simple method to distinguish between HO and high-valent metal intermediates, further kinetic experiments with both DMSO and methane, at varying concentration of either the nitroxide or the metal complex, could help discriminate further between the species. In this case, the product distribution in the two experiments is expected to vary, since under these conditions the oxidation of methane may be due to the HO whereas oxidation of DMSO may be by both HO and high valent metal species.

Appendix A. Supplementary information for Chapters II and III

A1 DMSO Titration

In order to determine saturated DMSO concentrations for the Fenton reactions, experiments were carried out in varying concentrations of DMSO at fixed concentrations of the nitroxide (3ap). In this case, the HO trapping using DMSO may be may be illustrated thus

$$\mathbf{OH} + \mathbf{DMSO} \xrightarrow{\mathbf{6.6 \times 10^9\ M^{-1}s^{-1}}} \mathbf{CH_3\text{-}3apf}$$

$$\mathbf{OH} + \mathbf{Si} \longrightarrow \mathbf{Products}$$

Where Si, denotes the sum of other HO competitors, including the nitroxide (3ap). Assuming that 3ap is the major HO competitor for HO, then, product II yield may be given by

$$\mathbf{Y_{DMSO}} = \frac{\mathbf{Y_o\, k_{DMSO}[DMSO]}}{\mathbf{K_{DMSO}[DMSO] + k_{3ap}[3ap]}} \qquad \text{A1}$$

Where, Y_o is the product yield associated with the total HO generated. At very high [DMSO], k_{DMSO} [DMSO] >>> k_{3ap} [3ap] and II yield reaches its maximum, i.e. $^{max}Y_{DMSO.}$ However, at half saturation value, $Y_{DMSO} = Y_o / 2$, the concentration of DMSO may be denoted as $[DMSO]_{½}$, and therefore,

$$k_{DMSO}[DMSO]_{1/2} = k_{3ap}[3ap] \qquad \text{A2}$$

or

$$[DMSO]_{1/2} = \frac{k_{3ap}[3ap]}{k_{DMSO}} \qquad \text{A3}$$

The experimental value of $[DMSO]_{½}$ may be obtained by a plot of Y_{DMSO} against [DMSO],(which predicts a parabolic behavior), whereas, the literature value may be obtained from known values of the rate constant of the reaction of HO with DMSO and 3ap (Table B2, Appendix B) . If the two values compare favorably, then free HO involvement is implied. In this study, when lower concentrations of 3ap (50 μM) were employed, an experimental $[DMSO]_{½}$ value of 45.0 μM was obtained (Figure A1, Appendix) which compared well with the predicted literature value of 37.0 μM.

At higher concentration of 3ap (500 μM), the experimental value of $[DMSO]_{½}$, (514 ± 136) is close (within experimental error) to the predicted literature value of (370.5 μM) (Figure A2, Appendix A). This also indicates that [DMSO] concentrations above 100 μM and above 700 μM represent maximum Y_o yields and indicate saturated DMSO conditions in experiments with low (50 μM) and higher (500 μM) 3ap concentrations respectively. It was further established that, under conditions of saturated DMSO, product II yield is independent of the concentration of the nitroxide (3ap), (Figure A3, Appendix A). Under conditions where the Fenton reaction was performed with otherwise similar concentrations of 3ap (50 μM or 500 μM), DMSO concentrations of 1.5 mM or 10 mM were employed to ensure saturating DMSO conditions.

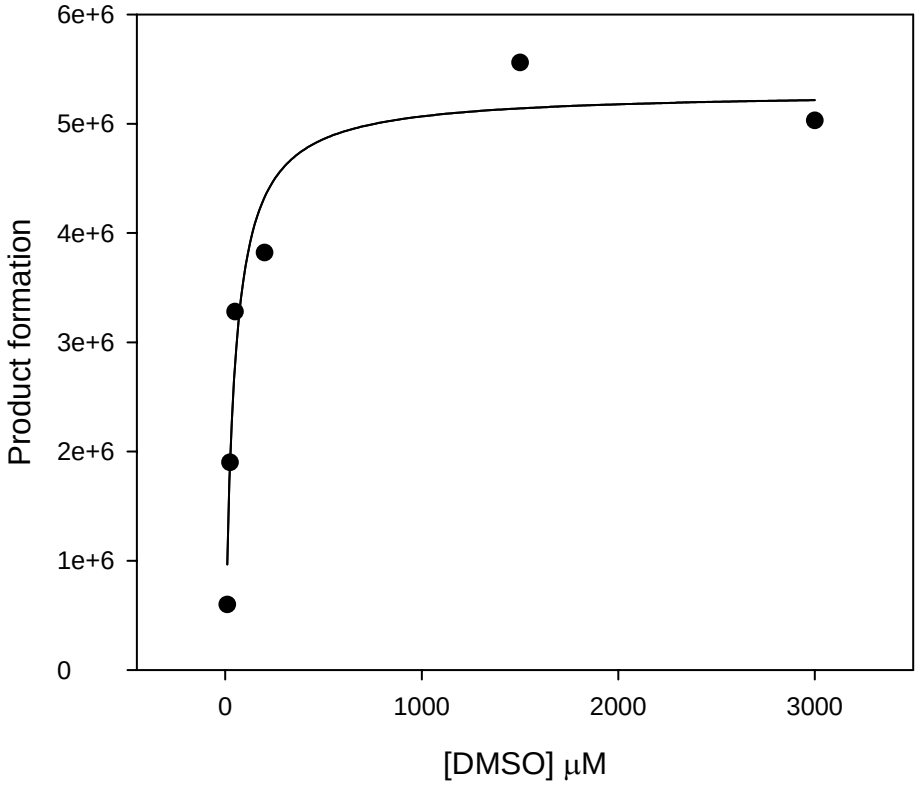


Fig A1. Dependence of II yield on [DMSO] at a fixed concentration of nitroxide. Reaction conditions; 50 μM 3ap, 150 μM KNO_3 at pH 7.4. The experiment was performed using irradiation a 300 W broadband lamp at wavelength above 300 nm. The concentration of DMSO was varied from 0.01 mM, 0.025 mM, 0.05 mM, 0.20 mM, and 1.5 mM to 3.0 mM.

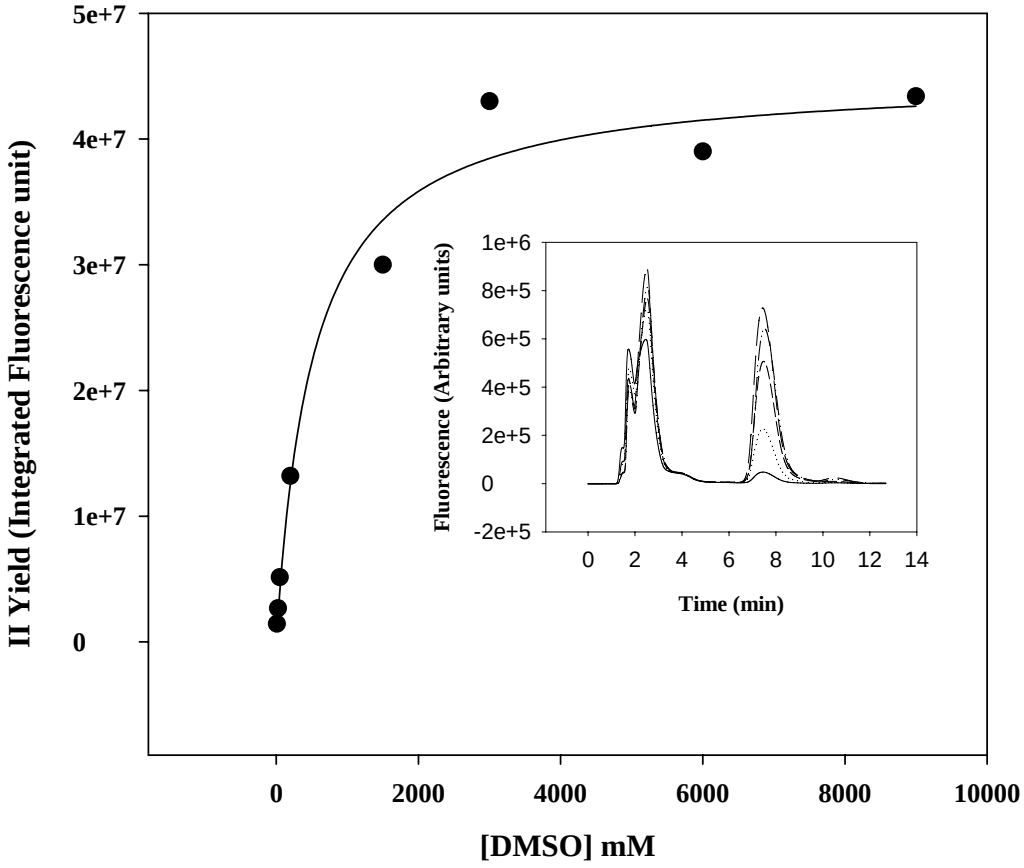


Fig A2. Dependence of II yield on [DMSO] at a fixed concentration of nitroxide. Reaction conditions; 500 μM 3ap, 150 μM KNO_3 at pH 7.4. The experiment was performed using a 300 W broadband irradiation lamp at wavelengths above 300 nm. The concentrations of 3ap used were, 0.01, 0.025 mM, 0.050mM, 0.20mM, 1.5 mM and 3.0 mM. Inset shows chromatograms of product II formation at varying [DMSO], 0.025 (———), 0.20 (……), 1.50 (– – –), 6.0 (– · · –) and 9.0 (—— ——) mM.

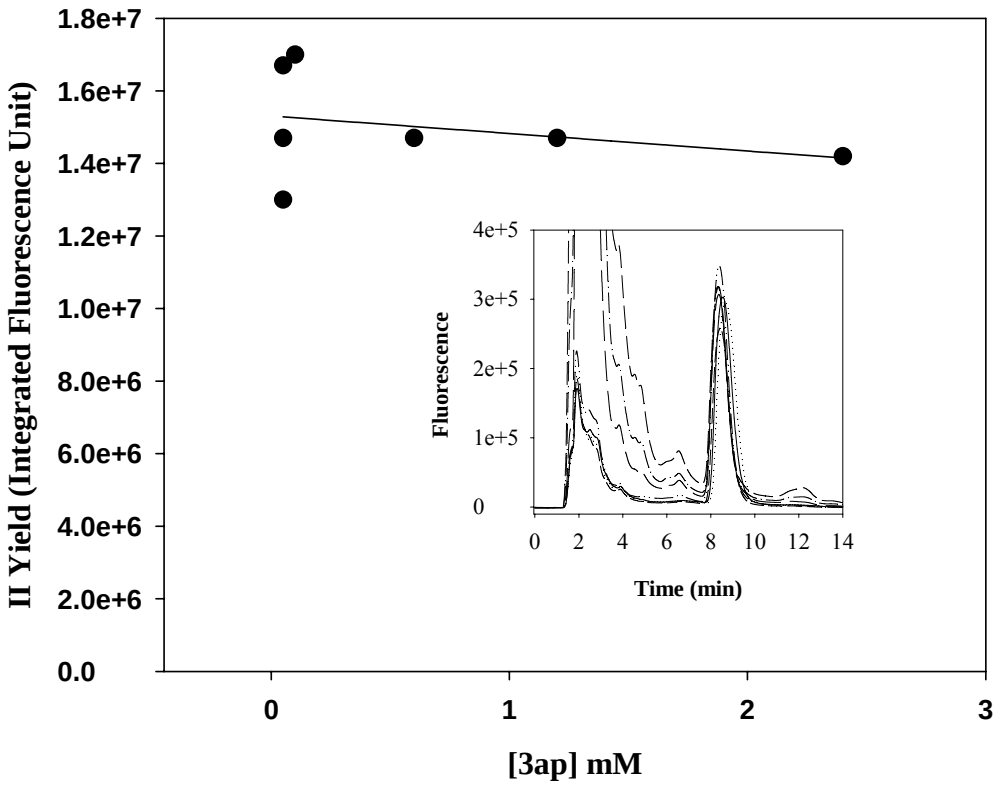


Figure A3. Dependency of II yield on 3ap at saturated [DMSO]. Other reaction conditions; 1.5 mM DMSO, 150 μM H_2O_2, 10 μM Fe-EDTA in 100 mM phosphate buffer at pH 7.4. Inset, shows chromatograms illustrating II formation at varying concentrations of 3ap; 0.05 mM (———), 0.10 mM (……), 0.60 mM (– – –), 1.2 mM (– · · –), and 2.4 mM(—— ——), at the fixed [DMSO] 1.5 mM. The first three chromatograms represent the lowest 3ap concentration (0.05 mM).

A2. Supplementary Chromatograms

Figures A4 to A10 present chromatograms for results obtained from a series of experiments under various conditions. These figures provide additional data that is presented elsewhere in the text. Figure A4 provides additional information for a competition study with benzoic acid in presence of DMSO (Figure 2.10, (Text). Figures A5 and A6 chromatographs depicting the dependency of II yield on metal ion concentration in the case of iron phosphate at pH 4.2 (Figure 2.13 (Text).

On the other hand, Figure A7 and A8 present chromatograms depicting II formation in the nitroxide competition experiments at neutral pH, Whereas Figure A9 presents chromatograms showing the dependency of II yield on metal ion formation at pH 7.4. Finally, Figure A10 provides additional information showing the dependency of II yield on concentration of the nitroxide in a Fenton reaction using Fe-NTA.

A3. Tables of Values and Fitting Parameters

The fitting parameters obtained from a fit of the experimental data to expression II.1 in the case of the nitrate competition studies are shown in Table A1. These parameters were used to compute k_{3ap}/k_{DMSO} values (Table 2.2, Text). Similarly, Table A2 shows fitting parameters obtained from a fit of experimental data to expression II.3 in the case of BA competition experiments and which were used to evaluate the k_{BA} values (Table 2.3, Text). On the other hand, Table A3 provides comparison values Y_{DMSO}/Y_{CH4} that were obtained from a series of experiments (Fenton reactions), with HO trapping using DMSO or methane. These values were used to obtain the average Y_{DMSO}/Y_{CH4} (Table 2.2 and 3.2, Text) values in the text under any given conditions.

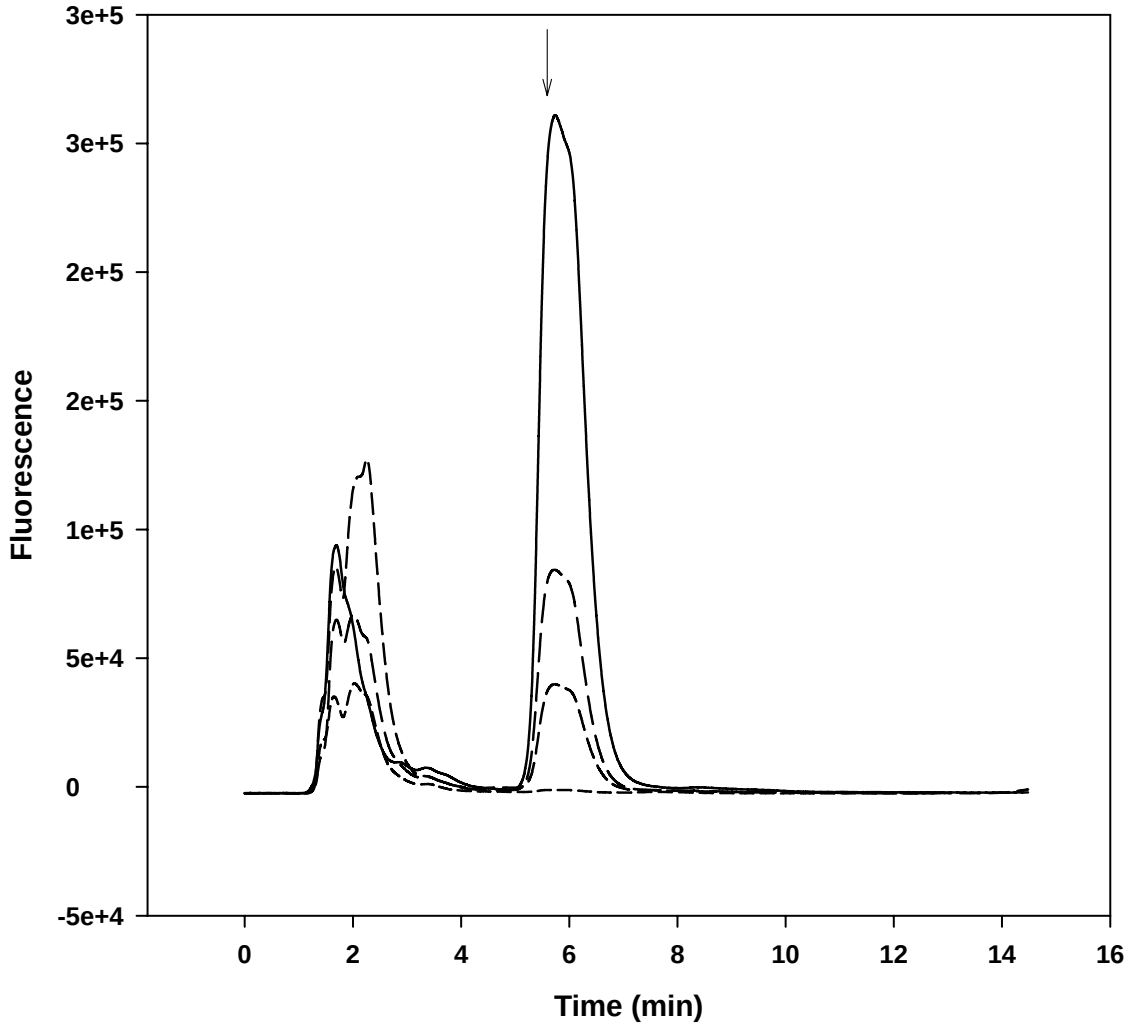


Figure A4. Chromatograms showing the decrease in II yield with increasing [BA] at fixed concentrations of DMSO and 3ap. Reaction conditions; [3ap] = 50 μM; [DMSO] = 50 μM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 4.2. The reactions were performed anaerobically at varying conditions whereby; (——) standard reaction without BA, (—— ——) standard reaction with 0.1 mM BA, (– – –) standard reaction with 1.0 mM BA and, (-----) standard reaction without Fe (II).

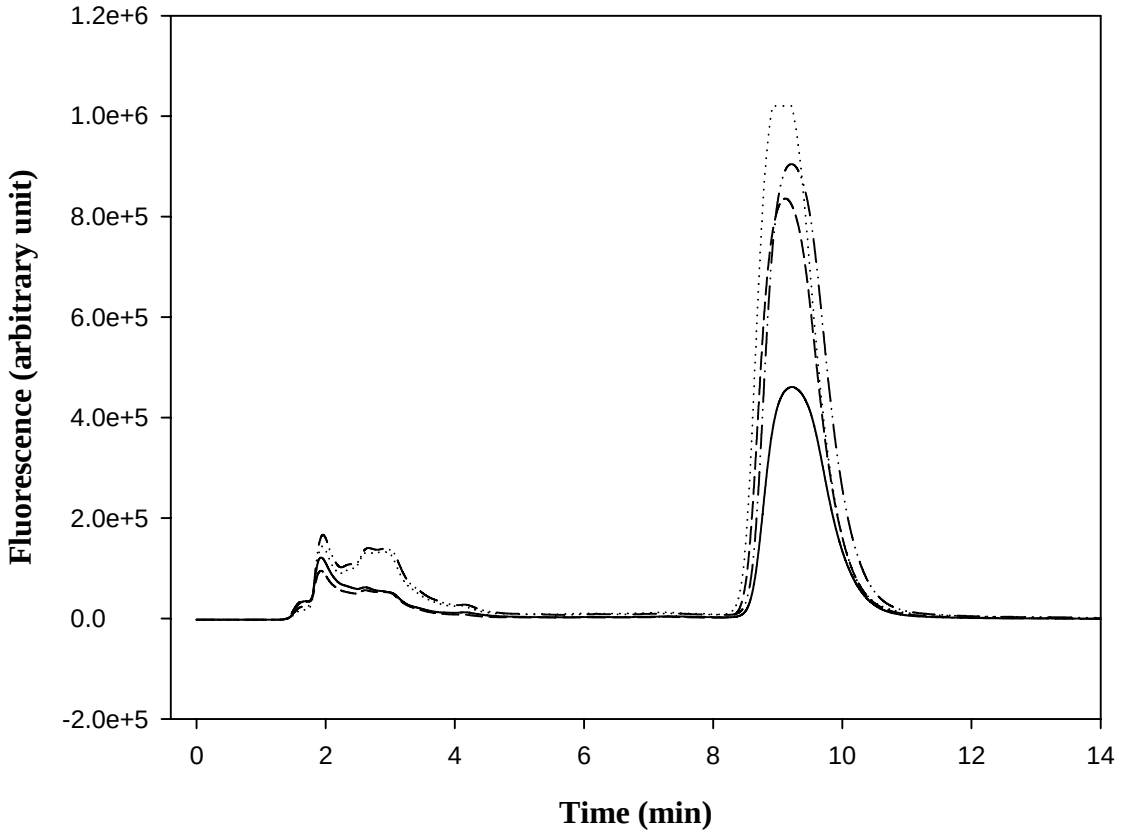


Figure A5. Chromatograms showing dependence of the yield of II on Fe (II) ions. Reaction conditions; [DMSO] = 10 mM, [3ap] = 50 μM, [H_2O_2] = 150 μM, in 2 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 μM Fe (II) ions and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of Fe (II); 5.0 μM, (——); 10.0 μM, (……); 17.5 μM, (— — —) and 25.0 μM, (— · · —). . The 10 μ M sample shown was further diluted twice, whereas the 17.5 μM and 25.0 μM samples were diluted three and four times respectively before injecting onto the HPLC to avoid saturating the detector.

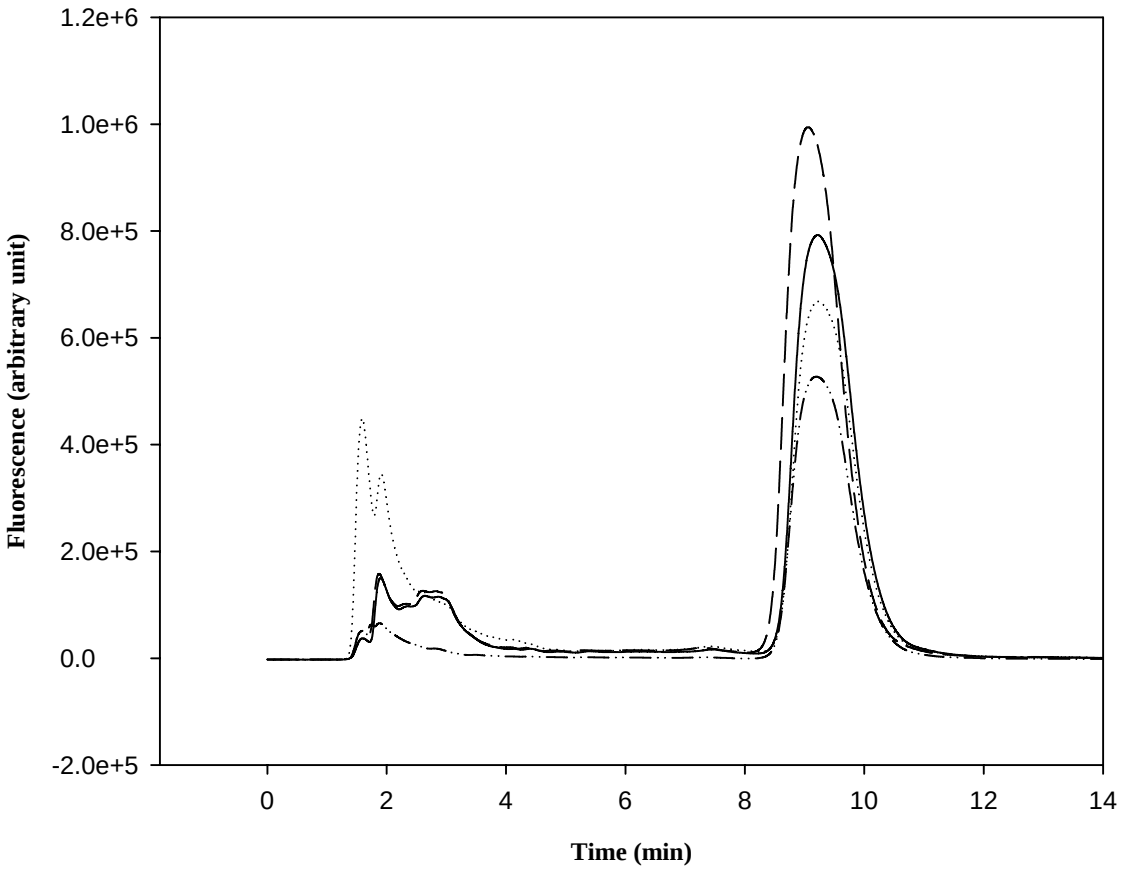


Figure A6. Chromatograms showing dependence of the yield of II on Fe (II) ions. Reaction conditions; [DMSO] = 10 mM, [3ap] = 50 µM, [H_2O_2] = 150 µM, in 100 mM phosphate buffer at pH 4.2. The reactions were initiated by addition of 10 µM Fe (II) ions and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of Fe (II); 5.0 µM, (——); 10.0 µM, (……); 17.5 µM, (— — —) and 25.0 µM, (— · · —). The 10 µM, 17.5 µM and 25.0 µM samples were diluted two, three and four times before injection onto the HPLC to avoid saturating the detector.

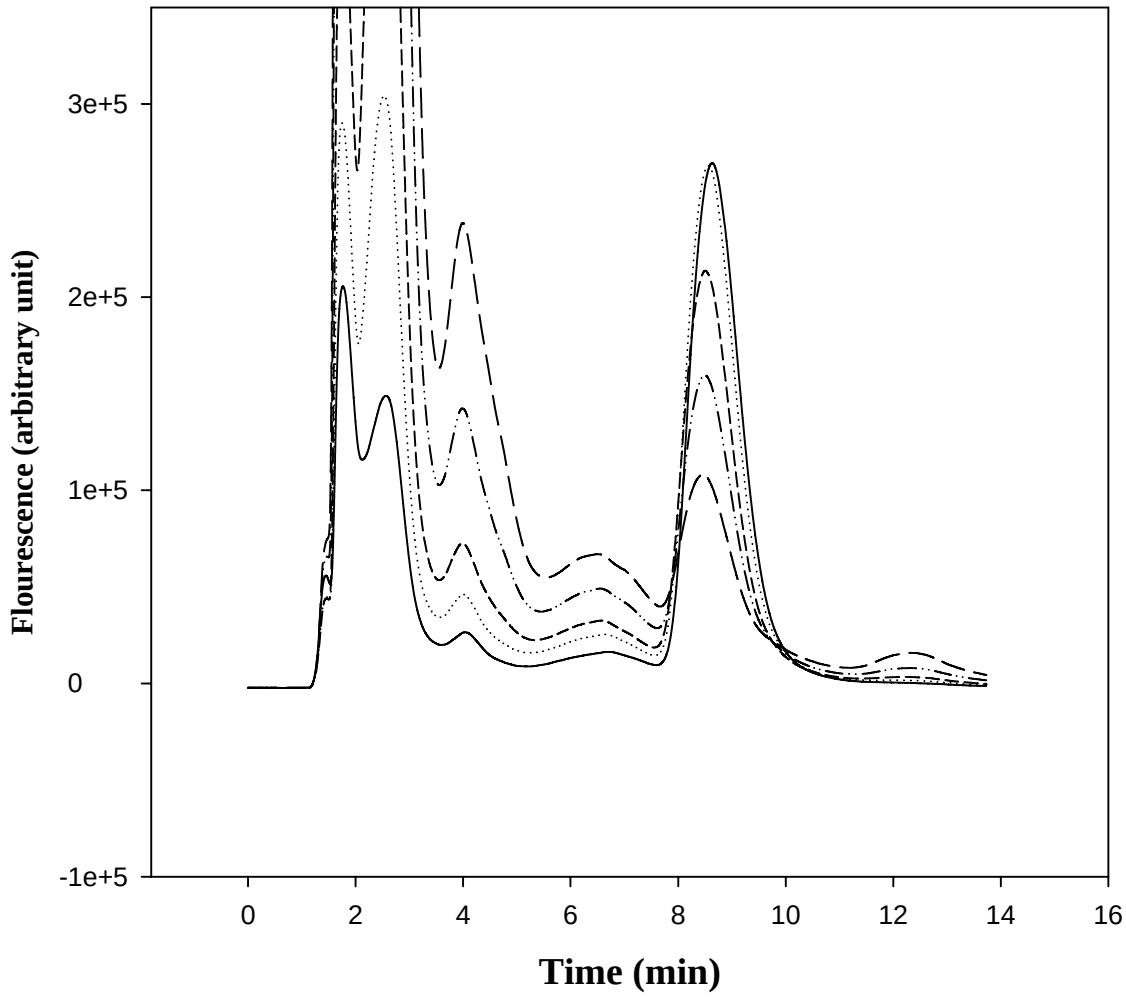


Figure A7. Chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. Reaction conditions; [DMSO] = 250 μM; [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 μM Fe (II) and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of 3ap; (——) 0.15 mM, (.....) 0.30 mM, (– – –) 0.60 mM, (– · · –) 1.2 mM, and (—— ——) 2.4 mM respectively.

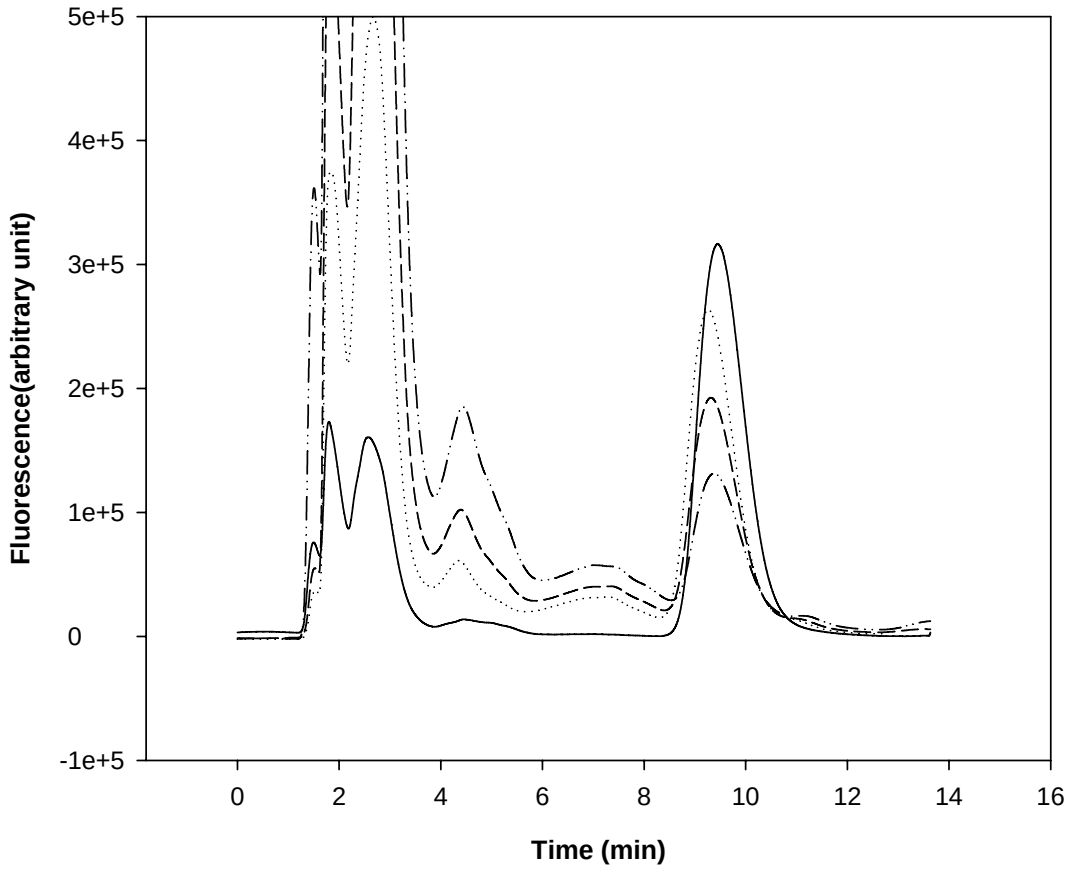


Figure A8. Chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. Reaction conditions; [DMSO] = 1.0 mM; [H_2O_2] = 150 µM, in 100 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 µM Fe (II) and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of 3ap; (——) 0.30 mM, (.....) 0.60 mM, (– – –) 1.2 mM, and (— · · —) 2.4 mM respectively.

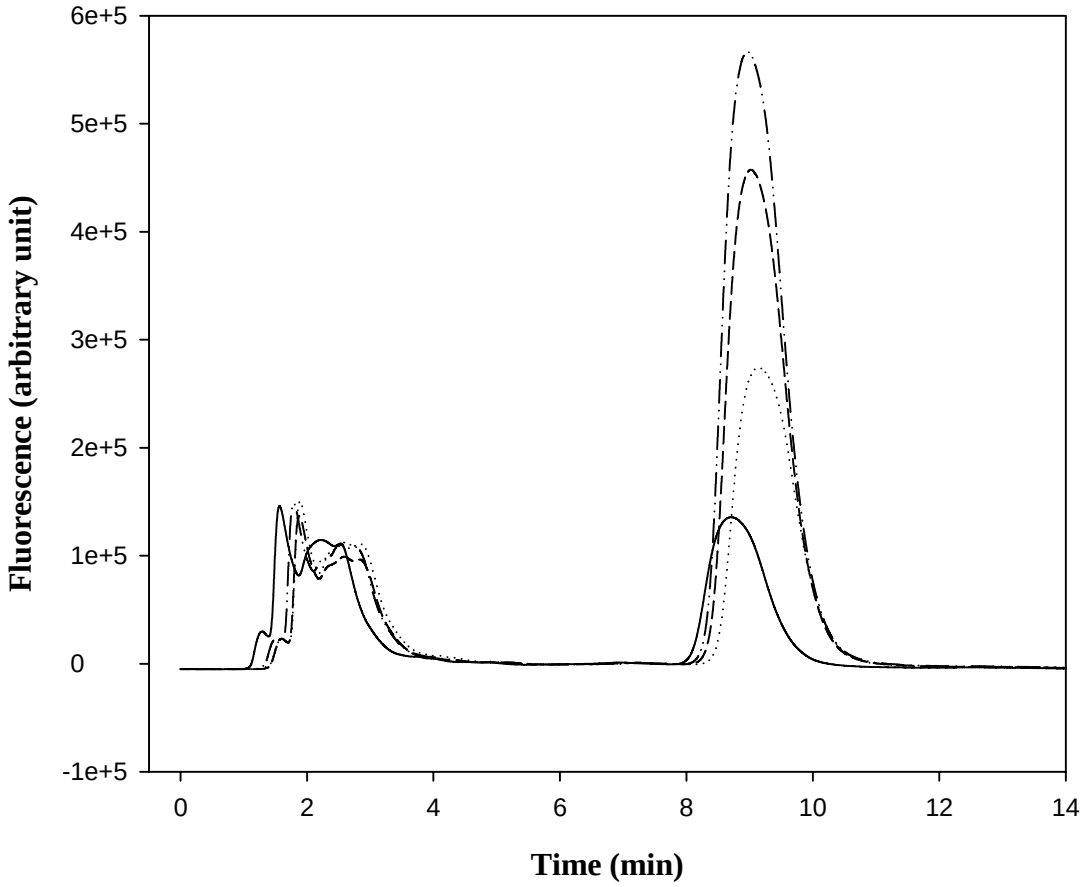


Figure A9. Chromatograms showing dependence of the yield of II on Fe (II) ions. Reaction conditions; [DMSO] = 10 mM, [3ap] = 50 μM, [H_2O_2] = 150 μM, in 100 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 μM Fe (II) ions and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of Fe (II); 5.0 μM, (——); 10.0 μM, (……); 17.5 μM, (— — —) and 25.0 μM, (— · · —).

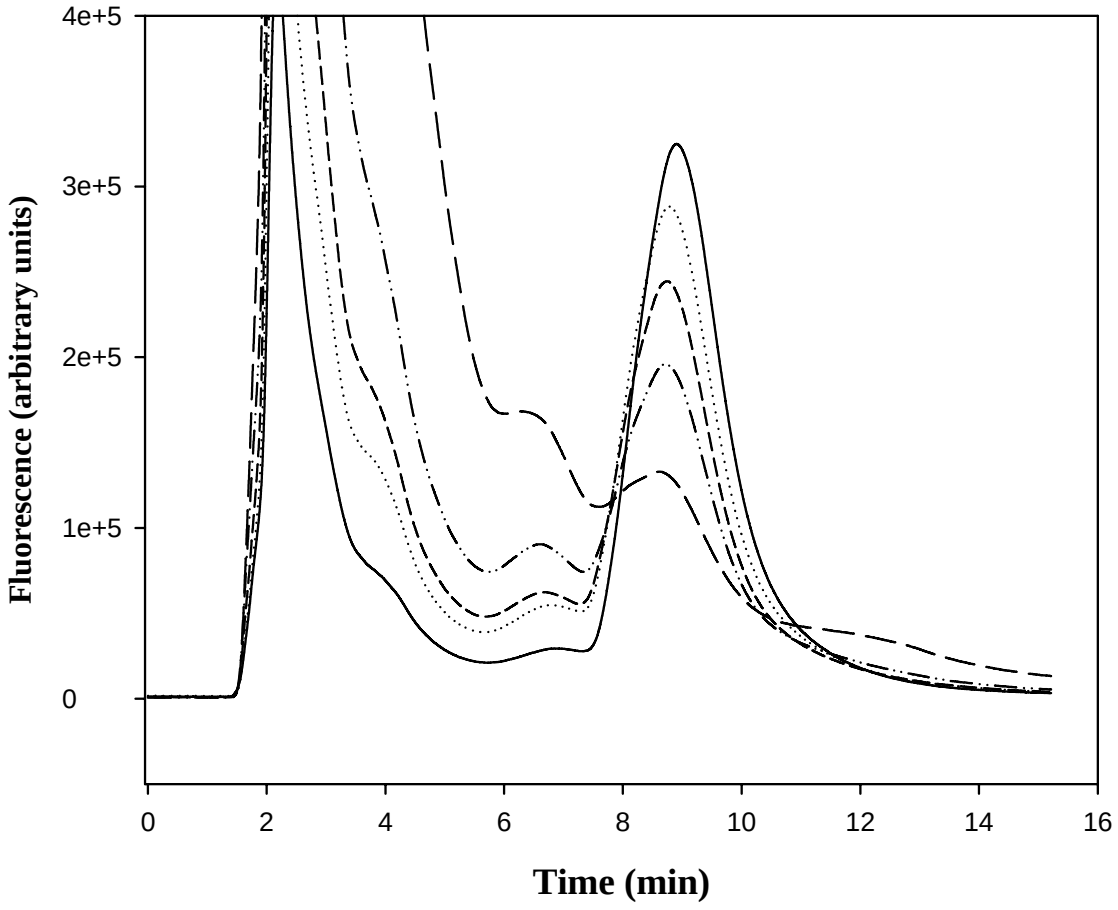


Figure A10. Chromatograms showing the effect of adding 3ap on II formation in presence of a constant concentration of DMSO. Reaction conditions; [DMSO] = 250 µM; [H_2O_2] = 150 µM, in 100 mM phosphate buffer at pH 7.4. The reactions were initiated by addition of 10 µM Fe (II)-NTA and allowed to continue anaerobically for 15 minutes. The experiments were carried with varying amounts of 3ap; (——) 0.10 mM, (.....) 0.30 mM, (– – –) 0.60 mM, (– · · –) 1.2 mM, and (—— ——) 2.4 mM respectively.

Table A1 3ap titration at constant [DMSO]

Experimental Study	Conditions [DMSO] µM	b* value	Standard error of b value
Nitrate	50 µM 75 µM 100 µM	1.61×10^4 1.34×10^4 6.60×10^3	2.8×10^3 1.1×10^3 1.0×10^3
pH 4.2	500 µM	1.93×10^3	4.3×10^2
pH 7.4	250 µM 500 µM 1000 µM 2000 µM	3.0×10^3 1.28×10^3 1.52×10^3 4.2×10^2	6.1×10^2 2.9×10^2 7.4×10^2 9.2×10^1
Fe-DTPA	300 µM	3.40×10^3	7.9×10^2
Fe-EDTA	150 µM	7.15×10^3	2.3×10^3
Fe-NTA	150 µM 300 µM	4.33×10^3 2.61×10^3	3.0×10^2 1.8×10^2

* The b values were obtained from a least squares fit of experimental data to expression II.1. They were used to calculate the ratio k_{3ap}/k_{DMSO} (Table 2.2). The standard error in the b values was obtained with a 95 % confidence. These errors were then computed in evaluating the k_{3ap}/k_{DMSO} ratio in the expression $k_{3ap}/k_{DMSO} = b$ [DMSO]. The error due to the concentration of DMSO was found to be negligible in comparison to that resulting from the fitting parameter 'b' and was therefore ignored in the calculation of the ratio.

Table A2 BA titration at constant [DMSO] or [CH4]

Experimental Study		Conditions [DMSO] µM	p value	Standard error of b value
DMSO	**pH 4.2**	30 µM 50 µM	9.6×10^3 5.08×10^3	1.45×10^3 1.63×10^3
	pH 7.4	30 µM	1.05×10^4	8.0×10^2
		$[CH_4]$ mM	**q value**	
CH_4	**pH 4.2**	1.5 mM	1.09×10^4	7.0×10^2
	pH 7.4	1.5 mM	1.42×10^4 1.53×10^4	7.0×10^2 9.2×10^4

* The values of p and q were obtained from a least squares fit of experimental data to expression II.1. These values were used to calculate the experimental value of the rate constant of the reaction of HO with BA (k_{BA}, Table 2.2). The standard error in the q and p values was obtained with a 95 % confidence. These errors were then computed in evaluating the k_{BA} value in the expression $k_{BA} = p \{(k_{DMSO} [DMSO] + k_{3ap} [3ap]\}$. The error due to the concentration of DMSO was found to be negligible in comparison to that resulting from the fitting parameters p and q and was therefore ignored in the calculation of k_{BA}.

Table A3: Product Comparison Y_{DMSO}/Y_{CH4}

Metal Complex	Y_{DMSO}/Y_{CH4} *	
	pH 4.2	pH 7.4
Fe-EDTA	3.6	4.0
	3.0	3.5
	3.3	2.6
Fe-DTPA	3.0	3.2
	3.5	4.0
	3.2	3.5
Fe-NTA	4.0	3.3
	3.0	2.7
	3.5	3.0
Fe-Phosphate	3.8	3.2
	3.0	3.9
	3.5	2.5

*The Y_{DMSO}/Y_{CH4} values were obtained from Fenton reactions performed under identical conditions but in presence of either DMSO or methane.

Appendix B. Supplementary Information, Rate constants, Binding constants, Reduction potentials and Speciation data Relevant to Chapters 2 and 3

Table B1 Rate Constants ($M^{-1}s^{-1}$) for the Oxidation of Fe-complexes by O_2, $O_2^{-\cdot}$, H_2O_2 and other species

LIGAND (L)	k_{-1} (O_2) $Fe^{2+} + O_2$	k_{-1} ($O_2^{-\cdot}$) $Fe^{3+} + O_2^{-\cdot}$	k_2 ($O_2^{-\cdot}$) $Fe^{2+} + O_2^{-\cdot}$	k_3 (H_2O_2) $Fe^{2+} + H_2O_2$	k_4 ($HO^{\cdot}$) $Fe^{2+} + HO$
EDTA	63 [a] 150 (7.6-10.7) [m] 270 (5-7) [l] 600 (7.0) [m]	1.0×10^6 1.3×10^6 (7.0) [f] 1.9×10^6 [j] 2.5×10^6 [d] 5.0×10^6 6.6×10^6(5.5) [a] 5.1×10^8	2.5×10^6 (4.7-7.3) [a] 1.2×10^8 [a] (acid pH) 5.0×10^8 [x] $\sim 10^6$ [m]	9.1×10^2 (6.0)[q] 7.0×10^3 (7.4)[s] 6.8×10^3 (4.7) [z] 7.0×10^3 (7.4)[r] 1.4×10^4 [w] 2.0×10^6 [e]	2.0×10^8[c] 2.0×10^9 [c] 5.0×10^9 (4.5)[x] 7.5×10^9 (6.2)x
DTPA	3.5 (7.5)[a] 7.0 [i] 13.5 (4.0)[a]	negligible 6×10^3 [f] $< 10^4$ [d] 5×10^4 (7.0) [a] $<10^5$ [n]	2.0×10^4 [a] $<10^5$ (7.5) [a] 2×10^7 [n]	4.1×10^2 [w] 5.1×10^2 (7.4)[r] 8.0×10^2 (7.4)[s] 1.37×10^3 [u] 1.35×10^4 (4.0)[t]	5.0×10^9 [c]
NTA	80			9.7×10^3 [t] 1.84×10^4 (6.0)[q] 3.04×10^4 [s]	2.3×10^9 [c] 5.0×10^9 [x] 2.5×10^9 (6.2)[x] 1.1×10^9 (11)[x]
Fe^{2+} (phosphate)	52 (2.4) [k] $\sim 10^3$ (7.4)[g]	1.5×10^8 [f]	1.0×10^7 [f]	41.5[p] 60[q] 75 [v] 2×10^2 [t] $\sim 10^3$ (7.4)[g] 2×10^4 [h]	3.3×10^6 3.5×10^8 [y]

Key equations

$$Fe(II)L + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe(III)L + O_2^{\dot{-}}$$

$$Fe(II)L + O_2^{\dot{-}} \xrightarrow{k_2} Fe(III) + O_2$$

$$Fe(II)L + H_2O_2 \xrightarrow{k_3} Fe(III)L + H\dot{O} + OH^-$$

$$Fe(II)L + HO^{\cdot} \xrightarrow{k_4} Fe(III)L + HO^-$$

Key references for Table B1

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Table B2. Rate Constants of the Reaction of HO with various substrates

Substrate	Rate constant, $k/M^{-1}s^{-1}$
$HO^{\cdot}$	5×10^9
H_2O_2	4.5×10^7, (pH 7.0) $(2.0\text{-}3.8) \times 10^7$, (pH 7.0-11.0)
Fe(III)	$8.5 \times 10^7\ M^{-1}s^{-1}$
Fe(IV)	$1 \times 10^{7\ bb}$
BA	1.8×10^9, 4.3×10^9 ($\leq$3), 5.7×10^9 ($\approx$7), 6.2×10^9
3ap	$4.89 \times 10^{9\ aa}$
DMSO	6.6×10^9
CH_4	1.2×10^8
EDTA	2.8×10^9 4.0×10^8 (4.0) 2.0×10^8 (9.0)
DTPA	2.3×10^9 5×10^9 (5.0)
NTA	2.3×10^9 (0 – 9)
HPO_4^{2-}	1.5×10^5
$H_2PO_4^-$	~2×10^4
H_3PO_4	4.2×10^4

Unless otherwise stated, values obtained from a compilation by Buxton et al 1988. (Buxton et al "Rates of hydroxyl radicals in aqueous solutions" *J. Phy. Rev. Data* 1988, 17). [aa] Value obtained from this study using nitrate photolysis, [bb] value from, Logager T.; Holman, J.; Sehested, K.; Pedersen, T. *Inorg. Chem.* 1992, 31, 3523-3529. The values in brackets indicate the pH conditions under which the values were measured.

Table B3 Binding Constants for Fe-Chelates Employed in this Study

Fe-Chelate	Logarithmic Stability Constant	
	K ($Fe^{II}L$)	K ($Fe^{III}L$)
Fe-EDTA	14.3	25.1
Fe-NTA	8.8	15.9
Fe-DTPA	16.0	27.5

Values obtained from Kurimura et al (Kurimura Y. Ochiai R., and Matsura N. "Oxygen oxidation of ferrous ions induced by chelation" *Bull. Chem. Soc. Japan.* **1968**, 41, 2234-2239.

Table B4. Reduction Potentials of Relevant Species

Half Cell	Standard reduction Potential , E^{0}'(V)
H_2O / $e^-_{(aq)}$	- 2.84
O_2, H^+ / HO_2	-0.46
O_2 / $O_2^{-\cdot}$	-0.33
Fe^{II}-DTPA / Fe^{III}-DTPA	0.03
$Fe^{III}_{(aq)}$ / Fe^{II}	0.11
Fe^{III}-EDTA / Fe^{II}-EDTA	0.12
H_2O_2, H^+ / H_2O, OH	0.32
$O_2^{-\cdot}$,$2H^+$ / H_2O_2	0.94
OH, H^+ / H_2O	2.31

Data corrected to a pH of 7.0, and therefore values obtained $E^{0'}$ instead of E^{o}. For each couple, the oxidized species is on the left and the reduced species is on the right. Data obtained from a compilation by Buettner et al 1993, (Buettner G.R. "The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol and ascorbate. *Arch. Biochem. Biophy*. 1993, 300, 535.

Table B5. Speciation information for the Fe-Complexes employed in study

Fe-COMPLEX	MAJOR SPECIES
Fe-Phosphate	Fe^{2+}, (pH 4.2); 46% [**2 mM**] $FeH_2PO_4^+$, (pH 4.2); 54% $FeH_2PO_4^+$, (pH 4.2); 95% $FeHPO_4$, (pH 7.4); 91% **100 mM**
Fe-EDTA	Fe-$EDTA^{-2}$, (pH 4.2); 90% H_2EDTA^{-2}, (pH 4.2); 3% Fe-$EDTA^{-2}$, (pH 7.4); 99%
F-DTPA	Fe-H-$DTPA^{-2}$, (pH 4.2); 84% FeOH-$DTPA^{-4}$, (pH 7.4); 74% Fe-$DTPA^{-3}$, (pH 7.4); 249%
Fe-NTA	Fe-NTA^-, (pH 7.4); 94%

In all cases 10 μM $FeSO_4$, used and for the ligand complexes, 2 mM phosphate buffer employed. Species obtained by calculation using V-Minteq model program supplied by NIST Version 3.2 (See attached tables for details on other minor species involved).

General reaction conditions in Tables A6-A13 were 10 μM FeSO4, at the appropriate pH and 2mM phosphate buffer except where indicated otherwise. Values were obtained by calculation using Vminteq program supplied by NIST Version 3.2.

Table B6. Fe-Phosphate Speciation, (in 2 mM Phosphate buffer at pH 4.2)

Speciation	Concentration	Activity	Log activity
$Fe(OH)_2$ (aq)	3.7302E-18	3.7311E-18	-17.428
$Fe(OH)_3^-$	1.9532E-24	1.8828E-24	-23.725
Fe^{+2}	**5.3657E-06**	**4.6334E-06**	**-5.334**
$FeH_2PO_4^+$	**4.5892E-06**	**4.4239E-06**	**-5.354**
$FeHPO_4$ (aq)	3.5294E-08	3.5303E-08	-7.452
$FeOH^+$	3.0535E-11	2.9435E-11	-10.531
$FeSO_4$ (aq)	9.7554E-09	9.7578E-09	-8.011
H^{+1}	6.5453E-05	6.3096E-05	-4.2
$H_2PO_4^-$	1.9763E-03	1.9051E-03	-2.72
H_3PO_4	1.6897E-05	1.6901E-05	-4.772
HPO_4^{-2}	2.2164E-06	1.9139E-06	-5.718
HSO_4^-	5.4877E-08	5.2900E-08	-7.277
OH^-	1.6554E-10	1.5958E-10	-9.797
PO_4^{-3}	1.7795E-14	1.2791E-14	-13.893
SO_4^{-2}	9.9354E-06	8.5794E-06	-5.067

Table B7. Fe-Phosphate Speciation, (in 100 mM Phosphate at pH 4.2)

Speciation	Concentration	Activity	Log activity
$Fe(OH)_2$ (aq)	1.5151E-19	1.5327E-19	-18.815
$Fe(OH)_3^-$	9.3896E-26	7.7085E-26	-25.113
Fe^{+2}	4.2180E-07	1.9161E-07	-6.718
$FeH_2PO_4^+$	**9.5164E-06**	**7.8126E-06**	**-5.107**
$FeHPO_4$ (aq)	6.1631E-08	6.2344E-08	-7.205
$FeOH^+$	1.4778E-12	1.2132E-12	-11.916
$FeSO_4$ (aq)	2.1049E-10	2.1293E-10	-9.672
H^{+1}	7.6855E-05	6.3096E-05	-4.2
$H_2PO_4^-$	9.9097E-02	8.1356E-02	-1.09
H_3PO_4	7.1349E-04	7.2175E-04	-3.142
HPO_4^{-2}	1.7992E-04	8.1731E-05	-4.088
HSO_4^-	3.4001E-08	2.7914E-08	-7.554
OH^-	1.9373E-10	1.5905E-10	-9.798
PO_4^{-3}	3.2244E-12	5.4624E-13	-12.263
SO_4^{-2}	9.9658E-06	4.5271E-06	-5.344

Table B8. Fe-Phosphate Speciation, (in 100 mM phosphate at pH 7.4)

Speciation	Concentration	Activity	Log activity
$Fe(OH)_2$ (aq)	1.7395E-13	1.8082E-13	-12.743
$Fe(OH)_3^-$	1.9123E-16	1.4413E-16	-15.841
Fe^{+2}	2.7887E-07	8.9992E-08	-7.046
$FeH_2PO_4^+$	9.5571E-07	7.2032E-07	-6.142
$FeHPO_4$ (aq)	**8.7640E-06**	**9.1101E-06**	**-5.04**
$FeOH^+$	1.1982E-09	9.0307E-10	-9.044
$FeSO_4$ (aq)	6.8578E-11	7.1287E-11	-10.147
H^{+1}	5.2820E-08	3.9811E-08	-7.4
$H_2PO_4^-$	2.1190E-02	1.5971E-02	-1.797
H_3PO_4	8.6000E-08	8.9397E-08	-7.049
HPO_4^{-2}	7.8800E-02	2.5429E-02	-1.595
HSO_4^-	1.6657E-11	1.2555E-11	-10.901
OH^-	3.3444E-07	2.5207E-07	-6.598
PO_4^{-3}	3.4319E-06	2.6935E-07	-6.57
SO_4^{-2}	1.0000E-05	3.2271E-06	-5.491

Table B9. Fe (II) EDTA Speciation at pH 4.2

Speciation	Concentration	Activity	Log activity
$EDTA^{-4}$	7.3780E-15	4.0865E-15	-14.389
$Fe(OH)_2$ (aq)	1.5010E-19	1.5014E-19	-18.824
$Fe(OH)_3^-$	7.8612E-26	7.5762E-26	-25.121
Fe^{+2}	2.1612E-07	1.8644E-07	-6.729
$FeEDTA^{-2}$	**9.0373E-06**	**7.7963E-06**	**-5.108**
$FeH_2PO_4^+$	1.8508E-07	1.7837E-07	-6.749
$FeHEDTA^-$	5.5966E-07	5.3937E-07	-6.268
$FeHPO_4$ (aq)	1.4230E-09	1.4234E-09	-8.847
$FeOH^+$	1.2290E-12	1.1844E-12	-11.926
$FeOHEDTA^{-3}$	5.3234E-11	3.8182E-11	-10.418
$FeSO_4$ (aq)	3.9254E-10	3.9263E-10	-9.406
H^{+1}	6.5469E-05	6.3096E-05	-4.2
H_2EDTA^{-2}	3.1369E-06	2.7061E-06	-5.568
$H_2PO_4^-$	1.9807E-03	1.9089E-03	-2.719
H_3EDTA^-	2.3194E-07	2.2354E-07	-6.651
H_3PO_4	1.6930E-05	1.6935E-05	-4.771
H_4EDTA (aq)	2.3080E-09	2.3086E-09	-8.637
H_5EDTA^+	4.7795E-12	4.6062E-12	-11.337
H_6EDTA^{+2}	2.6031E-16	2.2457E-16	-15.649
$HEDTA^{-3}$	3.1892E-08	2.2874E-08	-7.641
HPO_4^{-2}	2.2229E-06	1.9177E-06	-5.717
HSO_4^-	5.4888E-08	5.2899E-08	-7.277
OH^-	1.6558E-10	1.5958E-10	-9.797
PO_4^{-3}	1.7869E-14	1.2817E-14	-13.892
SO_4^{-2}	9.9447E-06	8.5791E-06	-5.067

Table B10. Fe-EDTA Speciation, at pH 7.4

Speciation	Concentration	Activity	Log activity
$EDTA^{-4}$	8.43E-10	7.33E-10	-9.135
$Fe(OH)_2$ (aq)	2.58E-18	2.58E-18	-17.588
$Fe(OH)_3^-$	2.09E-21	2.07E-21	-20.685
Fe^{+2}	1.32E-12	1.28E-12	-11.894
$FeEDTA^{-2}$	**9.92E-06**	**9.58E-06**	**-5.019**
$FeH2PO_4^+$	4.86E-16	4.82E-16	-15.317
$FeHEDTA^-$	4.22E-10	4.18E-10	-9.379
$FeHPO_4$ (aq)	6.09E-15	6.09E-15	-14.215
$FeOH^+$	1.30E-14	1.29E-14	-13.891
$FeOHEDTA^{-3}$	8.04E-08	7.44E-08	-7.129
$FeSO_4$ (aq)	3.03E-15	3.03E-15	-14.519
H^{+1}	4.02E-08	3.98E-08	-7.4
H_2EDTA^{-2}	2.00E-07	1.93E-07	-6.714
$H_2PO_4^-$	7.59E-07	7.53E-07	-6.123
H_3EDTA^-	1.02E-11	1.01E-11	-10.997
H_3PO_4	4.21E-12	4.21E-12	-11.375
H_4EDTA (aq)	6.56E-17	6.56E-17	-16.183
H_5EDTA^+	8.33E-23	8.26E-23	-22.083
H_6EDTA^{+2}	2.63E-30	2.54E-30	-29.595
$HEDTA^{-3}$	2.80E-06	2.59E-06	-5.587
HPO_4^{-2}	1.24E-06	1.20E-06	-5.921
HSO_4^-	3.79E-11	3.76E-11	-10.425
OH^-	2.55E-07	2.53E-07	-6.597
PO_4^{-3}	1.37E-11	1.27E-11	-10.896
SO_4^{-2}	1.00E-05	9.66E-06	-5.015

Table B11. Fe-DTPA Speciation at pH 4.2

Speciation	Concentration	Activity	Log activity
$DTPA^{-5}$	2.2785E-19	9.0361E-20	-19.044
$Fe(OH)_2$ (aq)	5.2483E-19	5.2496E-19	-18.28
$Fe(OH)_2$-$DTPA^{-5}$	6.7886E-19	2.6922E-19	-18.57
$Fe(OH)_3^-$	2.7489E-25	2.6491E-25	-24.577
Fe^{+2}	7.5588E-07	6.5191E-07	-6.186
Fe_2-$DTPA^-$	1.6612E-09	1.6009E-09	-8.796
Fe-$DTPA^{-3}$	1.8398E-07	1.3188E-07	-6.88
$FeH_2PO_4^+$	6.4699E-07	6.2349E-07	-6.205
FeH-$DTPA^{-2}$	**8.4031E-06**	**7.2472E-06**	**-5.14**
$FeHPO_4$ (aq)	4.9742E-09	4.9754E-09	-8.303
$FeOH^+$	4.2976E-12	4.1415E-12	-11.383
FeOH-$DTPA^{-4}$	4.2385E-10	2.345E-10	-9.63
$FeSO_4$ (aq)	1.372E-09	1.3724E-09	-8.863
H^{+1}	0.000065474	6.3096E-05	-4.2
H_2-$DTPA^{-3}$	7.596E-07	5.4448E-07	-6.264
$H_2PO_4^-$	0.0019802	0.0019083	-2.719
H_3-$DTPA^{-2}$	3.3904E-06	2.924E-06	-5.534
H_3PO_4	0.000016925	1.69E-05	-4.771
H_4-$DTPA^-$	2.58E-07	2.48E-07	-6.604
H_5-DTPA (aq)	2.6054E-09	2.606E-09	-8.584
H_6-$DTPA^+$	6.6382E-12	6.3971E-12	-11.194
H_7-$DTPA^{+2}$	1.4463E-15	1.2473E-15	-14.904
H-$DTPA^{-4}$	5.4082E-12	2.9922E-12	-11.524
HPO_4^{-2}	2.2228E-06	1.9171E-06	-5.717
HSO_4^-	5.4872E-08	5.28E-08	-7.277
OH^-	1.6559E-10	1.59E-10	-9.797
PO_4^{-3}	1.7875E-14	1.28E-14	-13.892
SO_4^{-2}	9.9438E-06	8.576E-06	-5.067

Table B12. Fe-DTPA Speciation at pH 7.4

Speciation	Concentration	Activity	Log activity
$DTPA^{-5}$	1.4972E-12	1.1082E-12	-11.955
$Fe(OH)_2$ (aq)	1.7852E-18	1.7852E-18	-17.748
$Fe(OH)_2$-$DTPA^{-5}$	1.5170E-11	1.1228E-11	-10.95
$Fe(OH)_3^-$	1.4452E-21	1.4279E-21	-20.845
Fe^{+2}	9.2597E-13	8.8244E-13	-12.054
Fe_2-$DTPA^-$	3.6409E-14	3.5973E-14	-13.444
Fe-$DTPA^{-3}$	**2.4397E-06**	**2.1892E-06**	**-5.66**
$FeH_2PO_4^+$	3.3369E-16	3.2970E-16	-15.482
FeH-$DTPA^{-2}$	7.9653E-08	7.5909E-08	-7.12
$FeHPO_4$ (aq)	4.1697E-15	4.1698E-15	-14.38
$FeOH^+$	8.9932E-15	8.8856E-15	-14.051
FeOH-$DTPA^{-4}$	**7.4805E-06**	**6.1701E-06**	**-5.21**
$FeSO_4$ (aq)	2.0643E-15	2.0643E-15	-14.685
H^{+1}	4.0293E-08	3.9811E-08	-7.4
H_2-$DTPA^{-3}$	2.9624E-06	2.6583E-06	-5.575
$H_2PO_4^-$	7.5449E-07	7.4547E-07	-6.128
H_3-$DTPA^{-2}$	9.4518E-09	9.0075E-09	-8.045
H_3PO_4	4.1727E-12	4.1728E-12	-11.38
H_4-$DTPA^-$	4.8959E-13	4.8373E-13	-12.315
H_5-DTPA (aq)	3.1959E-18	3.1960E-18	-17.495
H_6-$DTPA^+$	5.0099E-24	4.9500E-24	-23.305
H_7-$DTPA^{+2}$	6.3902E-31	6.0898E-31	-30.215
H-$DTPA^{-4}$	2.8070E-08	2.3153E-08	-7.635
HPO_4^{-2}	1.2455E-06	1.1869E-06	-5.926
HSO_4^-	3.7524E-11	3.7075E-11	-10.431
OH^-	2.5599E-07	2.5293E-07	-6.597
PO_4^{-3}	1.4011E-11	1.2573E-11	-10.901
SO_4^{-2}	9.9999E-06	9.5299E-06	-5.021

Table B13. Fe-NTA Speciation, at pH 7.4

Speciation	Concentration	Activity	Log activity
$Fe(NTA)_2^{-4}$	1.1800E-11	1.0572E-11	-10.976
$Fe(OH)_2$ (aq)	3.3167E-13	3.3167E-13	-12.479
$Fe(OH)_3^-$	2.6711E-16	2.6528E-16	-15.576
Fe^{+2}	1.6851E-07	1.6395E-07	-6.785
$FeH_2PO_4^+$	6.2570E-11	6.2141E-11	-10.207
FeHNTA (aq)	5.0054E-11	5.0054E-11	-10.301
$FeHPO_4$ (aq)	7.8592E-10	7.8592E-10	-9.105
FeNTA-	**9.8271E-06**	**9.7598E-06**	**-5.011**
FeOH+	1.6622E-09	1.6509E-09	-8.782
$FeOHNTA^{-2}$	1.4301E-09	1.3914E-09	-8.857
$FeSO_4$ (aq)	3.9152E-10	3.9152E-10	-9.407
H^{+1}	4.0085E-08	3.9811E-08	-7.4
H_2NTA^-	1.0907E-10	1.0833E-10	-9.965
H_2PO4^-	7.6147E-07	7.5626E-07	-6.121
H_3NTA (aq)	4.3125E-16	4.3125E-16	-15.365
H_3PO_4	4.2332E-12	4.2332E-12	-11.373
H_4NTA^+	1.7287E-22	1.7169E-22	-21.765
$HNTA^{-2}$	3.1671E-06	3.0813E-06	-5.511
HPO_4^{-2}	1.2377E-06	1.2041E-06	-5.919
HSO_4^-	3.8110E-11	3.7849E-11	-10.422
NTA^{-3}	4.1838E-09	3.9331E-09	-8.405
OH^-	2.5467E-07	2.5293E-07	-6.597
PO_4^{-3}	1.3568E-11	1.2755E-11	-10.894
SO_4^{-2}	9.9996E-06	9.7286E-06	-5.012

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